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(54) Title: PURIFIED POLYPORUS LACCASES AND NUCLEIC ACIDS ENCODING SAME

(57) Abstract

The present invention relates to isolated nucleic acid constructs containing a sequence encoding a *Polyporus* laccase, and the laccase proteins encoded thereby.

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PURIFIED *POLYPORUS* LACCASES AND NUCLEIC ACIDS ENCODING SAME

Field of the Invention

The present invention relates to isolated nucleic acid fragments encoding a fungal oxidoreductase enzyme and the purified enzymes produced thereby. More particularly, the invention relates to nucleic acid fragments encoding a phenol oxidase, specifically a laccase, of a basidiomycete, *Polyporus*.

Background of the Invention

Laccases (benzenediol:oxygen oxidoreductases) are multi-copper-containing enzymes that catalyze the oxidation of phenolics. Laccase-mediated oxidations result in the production of aryloxy-radical intermediates from suitable phenolic substrate; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Such reactions are important in nature in biosynthetic pathways which lead to the formation of melanin, alkaloids, toxins, lignins, and humic acids. Laccases are produced by a wide variety of fungi, including ascomycetes such as *Aspergillus, Neurospora,* and *Podospora,* the deuteromycete *Botrytis*, and basidiomycetes such as *Collybia, Fomes, Lentinus, Pleurotus, Trametes, Polyporus* and perfect forms of *Rhizoctonia*. Laccases exhibit a wide range of substrate specificity, and each different fungal laccase usually differs only quantitatively from others in its ability to oxidize phenolic substrates. Because of the substrate diversity, laccases generally have found many potential industrial

applications. Among these are lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, juice manufacture, phenol resin production, and waste water treatment.

Although the catalytic capabilities are similar, laccases made by different fungal species do have different temperature and pH optima, and these may also differ depending on the specific substrate. A number of these fungal laccases have been isolated, and the genes for several of these have been cloned. For example, Choi *et al.*(Mol. Plant-Microbe Interactions 5: 119-128, 1992) describe the molecular characterization and cloning of the gene encoding the laccase of the chestnut blight fungus, *Cryphonectria parasitica*. Kojima *et al.* (J. Biol. Chem. 265: 15224-15230, 1990; JP 2-238885) provide a description of two allelic forms of the laccase of the white-rot basidiomycete *Coriolus hirsutus*. Germann and Lerch (Experientia 41: 801,1985; PNAS USA 83: 8854-8858, 1986) have reported the cloning and partial sequencing of the *Neurospora crassa* laccase gene. Saloheimo *et al.*(J. Gen. Microbiol. 137: 1537-1544, 1985; WO 92/01046) have disclosed a structural analysis of the laccase gene from the fungus *Phlebia radiata*.

Attempts to express laccase genes in heterologous fungal systems frequently give very low yields(Kojima et al., *supra*; Saloheimo et al., Bio/Technol. 9: 987-990, 1991). For example, heterologous expression of *Phlebia radiata* laccase in *Trichoderma reesei* gave only 20 mg per liter of active enzyme in lab-scale fermentation(Saloheimo, 1991, *supra*). Although laccases have great commercial potential, the ability to express the enzyme in significant quantities is critical to their commercial utility. Previous attempts to express basidiomycete laccases in recombinant hosts have resulted in very low yields. The

present invention now provides novel basidiomycete laccases which are well expressed in *Aspergillus*.

Summary of the Invention

The present invention relates to a DNA construct containing a nucleic acid sequence encoding a *Polyporus* laccase. The invention also relates to an isolated laccase encoded by the nucleic acid sequence. Preferably, the laccase is substantially pure. By "substantially pure" is meant a laccase which is essentially (i.e.,≥90%) free of other non-laccase proteins.

In order to facilitate production of the novel laccase, the invention also provides vectors and host cells comprising the claimed nucleic acid sequence, which vectors and host cells are useful in recombinant production of the laccase. The sequence is operably linked to transcription and translation signals capable of directing expression of the laccase protein in the host cell of choice. A preferred host cell is a fungal cell, most preferably of the genus *Aspergillus*. Recombinant production of the laccase of the invention is achieved by culturing a host cell transformed or transfected with the construct of the invention, or progeny thereof, under conditions suitable for expression of the laccase protein, and recovering the laccase protein from the culture.

The laccases of the present invention are useful in a number of industrial processes in which oxidation of phenolics is required. These processes include lignin manipulation, juice manufacture, phenol polymerization and phenol resin production.

Brief Description of the Figures

Figure 1 shows the DNA sequence and translation of genomic clone 21GEN, containing LCC1 (SEQ ID NO. 1)

Figure 2 shows the DNA sequence and translation of genomic clone 23GEN, containing LCC2 (SEQ ID NO. 3)

Figure 3 shows the DNA sequence and translation of genomic clone 24GEN, containing LCC3 (SEQ ID NO. 5)

Figure 4 shows the DNA sequence and translation of genomic clone 31GEN, containing LCC4 (SEQ ID NO. 7)

Figure 5 shows the DNA sequence and translation of genomic clone 41GEN, containing LCC5 (SEQ ID NO. 9)

Figure 6 shows the structure of vector pMWR1

Figure 7 shows the structure of vector pDSY1

Figure 8 shows the structure of vector pDSY10

Figure 9 shows the pH profile of the laccase produced by pDSY2;(A) syringaldazine oxidation; (B) ABTS oxidation.

Figure 10 illustrates a comparison of the use of laccase vs. H_2O_2, with various dye precursors, in hair dyeing, as a measurement of DL*.

Figure 11 illustrates a comparison of the use of laccase vs. H_2O_2, with various dye precursors, in hair dyeing, as a measurement of Da*.

Figure 12 illustrates a comparison of the use of laccase vs. H_2O_2, with various dye precursors and modifiers, in hair dyeing, as a measurement of DL*.

Figure 13 illustrates a comparison of the wash stability of hair dyed with laccase vs. H_2O_2.

Figure 14 illustrates the light fastness of hair dyed with laccase vs. H_2O_2.

Detailed Description of the Invention

Polyporus pinsitus is a basidiomycete, also referred to as *Trametes villosa*. *Polyporus* species have previously been identified as laccase producers(Fahraeus and Lindeberg, Physiol. Plant. 6: 150-158, 1953). However, there has been no previous description of a purified laccase from *Polyporus pinsitus*. It has now been determined that *Polyporus*

pinsitus produces at least two different laccases, and the genes encoding these laccases can be used to produce relatively large yields of the enzyme in convenient host systems such as *Aspergillus*. In addition, three other genes which appear to code for laccases have also been isolated.

Initial screenings of a variety of fungal strains indicate that *Polyporus pinisitus* is a laccase producer. The production of laccase by *P. pinsitus* is induced by 2,5-xylidine. Attempts are first initiated to isolate the laccase from the supernatant of the induced strains. Anion exchange chromatography identifies an approximately 65 kD(on SDS-PAGE) protein which exhibits laccase activity. The enzyme is purified sufficiently to provide several internal peptide sequences, as well as an N-terminal sequence. The initial sequence information indicates the laccase has significant homology to that of *Coriolus hirsutus*, as well as to an unidentified basidiomycete laccase (Coll et al., Appl. Environ. Microbiol. 59: 4129-4135, 1993. Based on the sequence information, PCR primers are designed and PCR carried out on cDNA isolated from *P. pinsitus*. A band of the expected size is obtained by PCR, and the isolated fragment linked to a cellulase signal sequence is shown to express an active laccase in *A. oryzae*, but at low levels. One of the PCR fragments is also used as a probe in screening a *P. pinsitus* cDNA library. In this manner, more than 100 positive clones are identified. The positive clones are characterized and the ends of the longest clones sequenced; none of the clones are found to be full-length.

Further attempts to isolate a full length clone are made. A 5-6 kb BamHI size-selected *P. pinsitus* genomic library is probed with the most complete cDNA fragment isolated as described above. Initial screening identifies one clone 24GEN(LCC3) having homology to the cDNA, but which is not the cDNA-encoded laccase and also not full length.

Subsequent screening of a 7-8kb BamHI/EcoRi size-selected library indicates the presence of at least two laccases; partial sequencing shows that one, called 21GEN(LCC1), is identical to the original partial cDNA clone isolated, and the second,called 31GEN(LCC4) is a new, previously unidentified laccase. Secondary screenings of an EMBL4 genomic bank with LCC1 as probe identifies a class of clone containing the entire LCC1 insert as well as the 5' and 3' flanking regions. Screening of the EMBL bank with LCC3 identifies two additional clones encoding laccases which had not previously been identified,41GEN(LCC5) and 23GEN(LCC2) and which differed structurally from the other three clones LCC1, LCC3, and LCC4. The nucleic acid and predicted amino acid sequences of each of the laccases is presented in Figures 1-5, and in SEQ ID NOS. 1-10. A comparison of the structural organization of each of the laccases is presented in Table 2. The laccases are generally optimally active at acid pH, between about 4-5.5.

LCC1 is used to create expression vectors, which are in turn used to transform various species of *Aspergillus*. Transformation is successful in all species tested, although expression levels are highest in *Aspergillus niger*. Shake flask cultures are capable of producing 15 or more mg/liter of laccase, and in lab-scale fermentors, yields of over 300mg/liter are observed. This is a significant improvement over laccase levels observed previously with other laccases and other fungal host cells.

According to the invention, a *Polyporus* gene encoding a laccase can be obtained by methods described above, or any alternative methods known in the art, using the information provided herein. The gene can be expressed, in active form, using an expression vector. A useful expression vector contains an element that permits stable integration of the vector into the host cell genome or autonomous replication

of the vector in a host cell independent of the genome of the host cell, and preferably one or more phenotypic markers which permit easy selection of transformed host cells. The expression vector may also include control sequences encoding a promoter, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a signal sequence may be inserted prior to the coding sequence of the gene. For expression under the direction of control sequences, a laccase gene to be used according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can direct the transcription of the laccase gene, include but are not limited to the prokaryotic ß-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; and in Sambrook et al., Molecular Cloning, 1989.

The expression vector carrying the DNA construct of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will typically depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host

cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the laccase DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amy*L), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amy*M), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amy*Q), or the promoters of the *Bacillus subtilis* xylA and xylB genes. In a yeast host, a useful promoter is the eno-1 promoter. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* or *A. awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and *glaA* promoters.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the laccase of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter. The vector may further comprise a DNA sequence enabling the vector to

replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B.subtilis* or *B.licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Examples of *Aspergillus* selection markers include *amdS*, *pyrG*, *argB*, *niaD*, *sC*, *trpC* and *hygB*, a marker giving rise to hygromycin resistance. Preferred for use in an *Aspergillus* host cell are the *amdS* and *pyrG* markers of *A. nidulans* or *A. oryzae*. A frequently used mammalian marker is the dihydrofolate reductase (DHFR) gene. Furthermore, selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

It is generally preferred that the expression gives rise to a product which is extracellular. The laccases of the present invention may thus comprise a preregion permitting secretion of the expressed protein into the culture medium. If desirable, this preregion may be native to the laccase of the invention or substituted with a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions. For example, the preregion may be derived from a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase or proteinase gene from *Rhizomucor miehei*, the gene for the α-factor from *Saccharomyces cerevisiae* or the calf preprochymosin gene. Particularly preferred, when the host is a fungal cell, is the signal sequence for *A. oryzae* TAKA amylase, *A. niger* neutral amylase, the *Rhizomucor miehei*

aspartic proteinase signal, the *Rhizomucor miehei* lipase signal, the maltogenic amylase from *Bacillus* NCIB 11837, *B. stearothermophilus* α-amylase, or *B. licheniformis* subtilisin.

The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. Molecular Cloning, 1989).

The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the recombinant production of a enzyme of the invention. The cell may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The host cell may be selected from prokaryotic cells, such as bacterial cells. Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces*

murinus, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The host cell may also be a eukaryote, such as mammalian cells, insect cells, plant cells or preferably fungal cells, including yeast and filamentous fungi. For example, useful mammalian cells include CHO or COS cells. A yeast host cell may be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. Useful filamentous fungi may be selected from a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g. *F. oxysporum*, can be used as a host cell. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023. A suitable method of transforming *Fusarium* species is described by Malardier et al., 1989.

The present invention thus provides a method of producing a recombinant laccase of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture medium. The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the laccase of the invention. Suitable media are available from commercial suppliers or may be prepared according to published formulae (e.g. in catalogues of the American Type Culture Collection).

In a preferred embodiment, the recombinant production of laccase in culture is achieved in the presence of an excess amount of copper. Although trace metals added to the culture medium typically contain a small amount of copper, experiments conducted in connection with the present invention show that addition of a copper supplement to the medium can increase the yield of active enzyme many-fold. Preferably, the copper is added to the medium in soluble form, preferably in the form of a soluble copper salt, such as copper chloride, copper sulfate, or copper acetate. The final concentration of copper in the medium should be in the range of from 0.2-2mM, and preferably in the range of from 0.05-0.5mM. This method can be used in enhancing the yield of any recombinantly produced fungal laccase, as well as other copper-containing enzymes, in particular oxidoreductases.

The resulting enzyme may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like. Preferably, the isolated protein is about 90% pure as determined by SDS-PAGE, purity being most important in food, juice or detergent applications.

In a particularly preferred embodiment, the expression of laccase is achieved in a fungal host cell, such as *Aspergillus*. As described in detail in the following examples, the laccase gene is ligated into a plasmid containing the *Aspergillus oryzae* TAKA α-amylase promoter, and the *Aspergillus nidulans amdS* selectable marker. Alternatively, the *amdS* may be on a separate plasmid and

used in co-transformation. The plasmid (or plasmids) is used to transform an *Aspergillus* species host cell, such as *A. oryzae* or *A. niger* in accordance with methods described in Yelton et al. (PNAS USA 81: 1470-1474,1984).

It is of particular note that the yields of *Polyporus* laccase in the present invention, using *Aspergillus* as host cell are unexpectedly and considerably higher than has previously been reported for expression of other laccases in other host cells. It is expected that the use of *Aspergillus* as a host cell in production of laccases from other basidiomycetes, such as *Coriolus* or *Trametes*, will also produce larger quantities of the enzyme than have been previously obtainable. The present invention therefore also encompasses the production of such *Polyporus-like* laccases in *Aspergillus* recombinant host cells.

Those skilled in the art will recognize that the invention is not limited to use of the nucleic acid fragments specifically disclosed herein, for example, in Figures 1-5. It will also be apparent that the invention encompasses those nucleotide sequences that encode the same amino acid sequences as depicted in Figure 1-5, but which differ from the specifically depicted nucleotide sequences by virtue of the degeneracy of the genetic code. Also, reference to Figures 1-5 in the specification and the claims will be understood to encompass both the genomic sequence depicted therein as well as the corresponding cDNA and RNA sequences, and the phrases "DNA construct" and "nucleic acid sequences" as used herein will be understood to encompass all such variations. "DNA construct" shall generally be understood to mean a DNA molecule, either single- or double-stranded, which may be isolated in partial form from a naturally occurring gene or which has been modified to contain segments of DNA which are combined and juxtaposed in a manner which would not otherwise exist in nature.

In addition, the invention also encompasses other *Polyporus* laccases, including alternate forms of laccase which may be found in *Polyporus pinsitus* and as well as laccases which may be found in other fungi falling within the definition of *Polyporus* as defined by Fries, or synonyms thereof as stated in Long et al., 1994, ATCC Names of Industrial Fungi, ATCC, Rockville, Maryland. Identification and isolation of laccase genes from sources other than those specifically exemplified herein can be achieved by utilization of the methodology described in the present examples, with publicly available *Polyporus* strains. Alternately, the sequence disclosed herein can be used to design primers and/or probes useful in isolating laccase genes by standard PCR or southern hybridization techniques. Other named *Polyporus* species include, but are not limited to, *P. zonatus, P. alveolaris, P. arcularius, P. australiensis, P. badius, P. biformis, P. brumalis, P. ciliatus, P. colensoi, P. eucalyptorum, P. meridionalis, P. varius, P. palustris, P. rhizophilus, P. rugulosus, P. squamosus, P. tuberaster,* and *P. tumulosus* . Also encompassed are laccases which are synonyms, e.g., anamorphs or perfect states of species or strains of the genus *Polyporus*. Strains of *Polyporus* are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), e.g., ATCC 26721, 9385, 11088, 22084, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM),e.g., DSM 1021, 1023, and 1182; and Centraalbureau Voor Schimmelcultures (CBS), e.g., CBS 678.70, 166.29, 101.15, 276.31, 307.39, 334.49, and 332.49. The invention also encompasses any variant nucleotide sequence, and the protein encoded thereby, which protein retains at least about an 80% homology, preferably at least about 85%, and most preferably at least about 90-95% homology with any one of the amino acid sequences depicted

in Figures 2-5, and which qualitatively retains the laccase activity of the sequence described herein. Useful variants within the categories defined above include, for example, ones in which conservative amino acid substitutions have been made, which substitutions do not significantly affect the activity of the protein. By conservative substitution is meant that amino acids of the same class may be substituted by any other of that class. For example, the nonpolar aliphatic residues Ala, Val, Leu, and Ile may be interchanged, as may be the basic residues Lys and Arg, or the acidic residues Asp and Glu. Similarly, Ser and Thr are conservative substitutions for each other, as are Asn and Gln. It will be apparent to the skilled artisan that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active enzyme. Retention of the desired activity can readily be determined by conducting a standard ABTS oxidation method, such as is described in the present examples.

The protein can be used in number of different industrial processes. These processes include polymerization of lignin, both Kraft and lignosulfates, in solution, in order to produce a lignin with a higher molecular weight. Such methods are described in, for example, Jin et al., Holzforschung 45(6): 467-468, 1991; US Patent No. 4,432,921; EP 0 275 544; PCT/DK93/00217, 1992.

The laccase of the present invention can also be used for in-situ depolymerization of lignin in Kraft pulp, thereby producing a pulp with lower lignin content. This use of laccase is an improvement over the current use of chlorine for depolymerization of lignin, which leads to the production of chlorinated aromatic compounds, which are an environmentally undesirable by-product of paper mills. Such uses are described in, for example, Current opinion in

Biotechnology 3: 261-266, 1992; J. Biotechnol. 25: 333-339, 1992; Hiroi et al., Svensk papperstidning 5: 162-166, 1976.

Oxidation of dyes or dye precursors and other chromophoric compounds leads to decolorization of the compounds. Laccase can be used for this purpose, which can be particularly advantageous in a situation in which a dye transfer between fabrics is undesirable, e.g., in the textile industry and in the detergent industry. Methods for dye transfer inhibition and dye oxidation can be found in WO 92/01406, WO 92/18683, EP 0495836 and Calvo, Mededelingen van de Faculteit Landbouw-wetenschappen/Rijiksuniversitet Gent.56: 1565-1567, 1991; Tsujino et al., J. Soc. Chem.42: 273-282, 1991.

The laccase is particularly well-suited for use in hair dyeing. In such an application, the laccase is contacted with a dye precursor, preferably on the hair, whereby a controlled oxidation of the dye precursor is achieved to convert the precursor to a dye, or pigment producing compound, such as a quinoid compound. The dye precursor is preferably an aromatic compound belonging to one of three major chemical families: the diamines, aminophenols(or aminonaphthols) and the phenols. The dye precursors can be used alone or in combination. At least one of the intermediates in the copolymerization must be an ortho- or para-diamine or aminophenol(primary intermediate). Examples of such are found in Section V, below, and are also described in US Patent No. 3,251,742, the contents of which are incorporated herein by reference. In one embodiment, the starting materials include not only the enzyme and a primary intermediate, but also a modifier(coupler) (or combination of modifiers), which modifier is typically a meta-diamine, meta-aminophenol, or a polyphenol. The modifier then reacts with the primary intermediate in the presence of the laccase, converting it to a colored

compound. In another embodiment, the laccase can be used with the primary intermediate directly, to oxidize it into a colored compound. In all cases, the dyeing process can be conducted with one or more primary intermediates, either alone or in combination with one or more modifiers. Amounts of components are in accordance with usual commericial amounts for similar components, and proportions of components may be varied accordingly.

The use of this laccase is an improvement over the more traditional use of H_2O_2, in that the latter can damage the hair, and its use usually requires a high pH, which is also damaging to the hair. In contrast, the reaction with laccase can be conducted at alkaline, neutral or even acidic pH, and the oxygen needed for oxidation comes from the air, rather than via harsh chemical oxidation. The result provided by the use of the *Polyporus* laccase is comparable to that achieved with use of H_2O_2, not only in color development, but also in wash stability and light fastness. An additional commercial advantage is that a single container package can be made containing both the laccase and the precursor, in an oxygen free atmosphere, which arrangement is not possible with the use of H_2O_2.

The present laccase can also be used for the polymerization of phenolic or aniline compounds present in liquids. An example of such utility is the treatment of juices, such as apple juice, so that the laccase will accelerate a precipitation of the phenolic compounds present in the juice, thereby producing a more stable juice. Such applications have been described in Stutz, Fruit processing 7/93, 248-252, 1993; Maier et al., Dt. Lebensmittel-rindschau 86(5): 137-142, 1990; Dietrich et al., Fluss. Obst 57(2): 67-73, 1990.

Laccases such as the *Polyporus* laccase are also useful in soil detoxification (Nannipieri et al., J. Environ. Qual.

20: 510-517,1991; Dec and Bollag, Arch. Environ. Contam. Toxicol. 19: 543-550, 1990).

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

I. ISOLATION OF A *POLYPORUS PINISITUS* LACCASE ENZYME

MATERIALS AND METHODS

1. Enzymatic assays

Unless otherwise stated, throughout the examples, laccase activity is determined by syringaldazine and 2,2'-bisazino(3-ethylbenzthiazoline-6-sulfonic acid)(ABTS), as follows. The oxidation of syringaldazine is monitored at 530 nm with 19 µM substrate. In 25 mM sodium acetate, 40 µM cupric sulfate, pH 5.5, at 30°C, the activity is expressed as LACU(µmole/min). For pH profile studies, Britton & Robinson(B&R) buffers are used, and are prepared according to the protocol described in Quelle, Biochemisches Taschenbuch, H.M. Raven, II. Teil, S.93 u. 102, 1964. ABTS oxidation is carried out with 1mM ABTS in 0.1 M NaAc, pH 5.0 at room temperature by monitoring either ΔAbs_{405} in a 96-well plate(Costar) or ΔAbs_{418} in a quartz cuvette. The overlay ABTS oxidase activity assay is carried out by pouring cooled ABTS-agarose(0.03-0.1 g ABTS, 1 g agarose, 50 ml H_2O, heated to dissolve agarose) over a native IEF gel or PAGE and incubating at room temperature.

2. Initial isolation of laccase

In order to isolate the laccase, 800 ml of culture fluid is filtered by HFSC on a Supra filter(slow filtering). The clear filtrate is then concentrated and washed on an Amicon cell with a GR81 PP membrane to a volume of 72 ml.

One ml aliquots of laccase are bound to a Q-sepharose HP(Pharmacia, Sweden) column, equilibrated with 0.1 M phosphate, pH7 and the laccase is eluted with a NaCl gradient. In all, 10 x 1 ml samples are purified, pooled,

concentrated and washed by ultrafiltration using a membrane with a molecular weight cut-off of 6kD.

3. Secondary purification

In a second purification, a fermentation broth is filtered and concentrated by ultrafiltration. The starting material contains 187 LACU/ml. The concentrate is quick-filtered on a Propex 23 filter(P & S Filtration), with 3% Hyflo Cuper-Cel(HSC; Celite Corporation), followed by two ultrafiltration on a Filtron filter with two membranes, each with a molecular weight cutof of 3 kD. The resulting sample (2.5 mS/cm, pH 7.0, at 4°C) is applied to a 130 ml Q-Sepharose column, equilibrated with sodium phosphate, 1.1 mS/cm, pH 7.0. Under these conditions the laccase does not bind to the column, but elutes slowly from the column during the application and wash with the equilibration buffer, resulting in a partial separation from other brownish material.

This partially purified preparation of 1.0mS, pH 7.0 at 20°C is applied to a Q-sepharose column. The column is equilibrated with 20mM sodium phosphate, 2.2 mS, pH 7.0. Under these conditions, the laccase binds to the column and is eluted by a gradient of 0-1 M NaCl over 20 column volumes.

3. Sequencing

For internal peptide sequencing, the purified protein is digested with trypsin, followed by peptide purification with HPLC. Purified peptides are sequenced in an Applied Biosystems 473A sequencer.

B. RESULTS AND DISCUSSION

1. Initial characterization

Total yield of the initial purification is about 50 mg(estimated at A280nm). The purified enzyme has a rich blue color, and appears as only two very close bands on SDS-PAGE at about 65 kd. A native PAGE overlaid with substrate

shows that both bands have laccase activity with ABTS. The absorption spectrum shows that besides an absorption at A280nm, the purified laccase also shows absorption at about 600nm.

2. Sequencing

A N-terminal determination of the protein initially purified shows a single sequence:

Gly-Ile-Gly-Pro-Val-Ala-Asp-Leu-Thr-Ile-Thr-Asn-Ala-Ala-Ala-Val-Ser-Pro-Asp-Gly-Phe-Pro...

Since the N-terminal sequence is not the ideal sequence for constructing a probe, additional experiments with a trypsin digest are conducted, followed by further purification(described above) and sequencing of fragments

2. Secondary purification and characterization

In the second purification, the second Q-Sepharose chromatographic step yields the following pools:

Q-Sepharose-2-pool-1 40 ml 112 LACU 47 LACU/A_{280}

Q-Sepharose-2-pool-3 80 ml 385 LACU 65 LACU/A_{280}

The elution yields >80% of the applied amount. The highly purified preparation Q-Sepharose-2-pool-3 has an A_{280} = 5.9, and A_{280}/A_{260} = 1.4. The purity of the laccase in the starting material is extremely high on a protein basis but the starting material is a very dark brown color. In SDS-PAGE, a double band is seen, with a dominating 65 kD band and a smaller 62 kD band. By anionic chromatography, only the dominating band is seen in the first peak(Q-Sepharose-2-pool-1), whereas both bands are seen in the second peak(Q-Sepharose-2-pool-3).

3. Sequence

A number of internal peptide sequences are determined, and compared with the *Coriolus hirsutus(Ch)* laccase sequence. The identified fragments are as follows:

Tryp 13:

Ser-Pro-Ser-Thr-Thr-Thr-Ala-Ala-Asp-Leu

Tryp 14:

Ser-Ala-Gly-Ser-Thr-Val-Tyr-Asn-Tyr-Asp-Asn-Pro-Ile-Phe Arg

Tryp 16:

Sequence 1:

Ser-Thr-Ser-Ile-His-Trp-His-Gly-Phe-Phe-Gln-Lys

Sequence 2:

Gly-Ile-Gly-Pro-Val-Ala-Asp-Leu-Thr-Ile-Thr-Asn-Ala-Ala-Val

Tryp 18:

Gly-Ile-Gly-Pro-Val-Ala-Asp-Leu-Thr-Ile-Thr-Asn

Tryp 19:

Sequence 1:

Leu-Gly-Pro-Ala-Phe-Pro-Leu-Gly-Ala-Asp-Ala-Thr-Leu-Ile-

Sequence 2:

Phe-Gln-Leu-Asn-Val-Ile-Asp-Asn-Asn-Thr-Thr-His-Thr-Met

Tryp 25:

Tyr-Ser-Phe-Val-Leu-Glu-Ala-Asn-Gln-Ala-Val-Asp-Asn-Tyr-Trp-Ile-Arg

Tryp 27

Gly-Thr-Asn-Trp-Ala-Asp-Gly-Pro-Ala-Phe

II. ISOLATION OF A *POLYPORUS PINISITUS* LACCASE CDNA CLONE

A. MATERIALS AND METHODS

1. RNA preparation

RNA is isolated from 10 grams of *P. pinsitus* mycelium grown under xylidine induction for 6.5 hours, using the guanidium/CsCl cushion method. The RNA is poly-A selected on an oligo-dT column, using standard conditions. 120µg mRNA is obtained and stored as lyophilized pellet in 5µg aliquots at -80°C.

2. Single stranded cDNA

Single stranded cDNA is synthesized using the reverse transcriptase "Super Script" (BRL) according to manufacturer's directions.

3. Construction of cDNA library

A cDNA library is constructed using the librarian IV cDNA kit (Invitrogen). Fifty cDNA pools, each containing approximately 5000 individual transformants, are obtained.

4. PCR

PCR is conducted under the following standard conditions: 100pmol of each primer, 10µl 10X PCR buffer(Perkin-Elmer), 40µl dNTP 0.5 mM, 2µl single stranded cDNA(or approximately 100 ng chromosomal DNA or 100 ng PCR fragment), H_2O to 100 µl, 2.5U Taq polymerase. The cycles are 3x(40°C/two minutes, 72°C/two minutes, 94°C/one minute) followed by 30x(60°C/two minutes, 72°C/two minutes, 94°C/1 minute).

B. RESULTS AND DISCUSSION

1. Cloning of *Polyporus pinsitus* laccase

PCR is carried out with the primer #3331:

ACCAGNCTAGACACGGGNTC/AGATACTG/ACGNGAGAGCGGAC/TTGCTGGTC ACTATCTTCGAAGATCTCG

and primer #3332:

CGCGGCCGCTAGGATCCTCACAATGGCCAA/CTCTCTG/CCTCG/ACCTTC.

A clear band of about 1500bp is obtained. The DNA is digested with NotI/HindIII, and fractionated on an agarose gel. The upper band(fragment #42) is purified and cloned into the *Aspergillus* vector pHD423. No transformants are obtained. Several attempts are carried out in order to clone the fragment, including redigestion with the restriction enzymes, phosphorylation of the ends, filling in with klenow and blunt-end cloning in SmaI cut puC18, without success. Hybridization with a laccase probe based on the laccase described in Coll et al., *supra*, indicates that the PCR product could be the *P. pinsitus* laccase. In a new attempt to clone the PCR fragment, a new PCR reaction is carried out, using the same conditions as for fragment #42. Again the result is a fragment of about 1500 bp(fragment #43). This time the fragment is cut with HindIII/BamHI, and

ligated to HindIII/BamHI-cut pUC18. Three clones, #43-/A,-B,-G are found to contain a fragment of 1500 bp. Partial sequencing reveals that these fragments are laccase related.

2.Expression of *Polyporus pinsitus* laccase

To express the laccase, the fragment #43 is joined to a signal sequence from a 43kD cellulase. The primer pHD433 (TAGCGGATCCCACAATGCGTTCCTCCCCCTCCTCCCGTCCGCCGTTGTGGCCGCCCTG CCGGTGTTGGCCCTTGCCGGCATTGGGCCCGTCGCGGACC) is used in a standard PCR reaction with a pUC forward primer(New England Biolabs). All three clones are used as templates in order to minimize the risk of working with DNA containing errors.

The PCR generated DNA from the reaction with a primer pHD433 and template 43-A and 43-G is cut with HindIII/BamHI and cloned into the *Aspergillus* expression vector pHD414(described in detail below). Several transformants are obtained.

Clones pHD433/43A-1,2, pHD433/43G-2,-3 are transformed into *A. oryzae*. The transformants from each transformation (between 3-10) are analyzed for laccase production. Activity is only obtained with pHD433/43G-3. The positive transformants (numbers 1, 4, 6) are reisolated on amdS plates, and retested. In an additional transformation round a further ten transformants are obtained with pHD433/43G-3. The clones #20, 23, 26, 28, and 29 are positive. The clones are reisolated and two single isolates are analyzed for laccase expression semiquantitatively by color development in an ABTS assay at pH 4.5. On a scale of +-+++, several clones show moderate to strong expression of laccase.

Further cloning is conducted to identify a full length clone. A xylidine-induced cDNA library consisting of approximately 350,000 transformants is screened using fragment #42-4 as a probe. More than 100 positive clones are detected. The clones are purified, rescreened, and analyzed on Southern blots. Two of the longest clones are

further characterized by DNA sequence determination. The longest clones are found to be identical and found to contain a poly-A stretch in the 3'end and to start at the amino acid number 4 in the amino terminus. A partial DNA sequence is determined from different clones.

pHD433/43G-3 is then used in further cloning studies as described in the following Section IV.

III. PURIFICATION AND CHARACTERIZATION OF ADDITIONAL *POLYPORUS PINSITUS* LACCASE WILD-TYPE ENZYMES

A. MATERIALS AND METHODS

1.Culture conditions

Shake flasks(250 ml medium/2.8 l baffled flask)are inoculated wtih several agar plugs taken from a week-old PDA plate of *P. pinsitus*. The medium contains, per liter, 10 g glucose, 2.5 g L-asparagine, 0.2 g L-phenylalanine, 2.0 g yeast extract, 2.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 2.0 mlAMG trace metals, 0.002 g $CuSO_4 \cdot 7H_2O$, 1.0 g citric acid, made with tape water, pH 5.0 before autoclaving. The cultures are grown at 18-22°C on a rotary shaker with low agitiation (~100 rpm). After 7 days, the pH of each shake flask is adjusted to ~6.0 by the addition of 0.25 ml 5 N NaOH and the cultures are induced by adding 0.5 ml of a 2,5-xylidine stock solution(xylidine diluted 1:10 into ethanol) to each flask. Flasks are incubated for an additional 24 hours, at which time the culture supernatant from each flask is recovered.

2. Materials.

Chemicals used as buffers are commercial products of at least reagent grade. Endo/N-glucosidase F is from Boehringer-Mannheim. Chromatography is performed on Pharmacia FPLC. Spectroscopic assays are conducted on either a spectrophotometer(Shimadzu PC160) or a microplate reader(Molecular Devices).

3.Purification

Culture broth is filtered first on cheesecloth and centrifuged at 1000 x g to remove gelatinous pinkish xylidine polymer. The supernatant is then filtered on Whatman #2 paper and concentrated from 1500 to 250 ml on S1Y100(Amicon, Spiral concentrator) at 4°C. The concentrated broth is diluted with water until it reaches 0.8 mS(from 2.5 mS) and then concentrated on S1Y100 to 250 ml. The washed broth, thawed from -20°C freezing overnight, is subjected to Whatman #2 paper filtration to remove residual pinkish material, and then pH adjusted by NaOH from pH 6.1 to pH 7.7. This yellowish broth, 275 ml with 0.8 mS, is applied on a Q-Sepharose XK-26 column(~64 ml gel) equilibrated with 10 mM Tris-HCl, pH 7,7, 0.7 mS. The first active laccase fraction runs through during loading and washing by the equilibrating buffer. The elution is carried out by a linear gradient of 0-0.5 M NaCl in the equilibrating buffer over 8.8 bed-volume. The second and third active fractions are eluted around 0.15 and 0.35M NaCl, respectively. No more active fractions are detected when the column is washed sequentially with 2 M NaCl and with 1 mM NaOH. The active fractions are pooled, adjusted to ~10mS, concentrated on Centricon-10(Amicon), and then applied onto Superdex 75(HR10/30, 24 ml, Pharmacia) equilibrated with 10mM Tris-HCl, 0.15 M NaCl, pH 8, 14 mS. During elution with the application buffer, laccase fractions are eluted off using the same elution volume for all three Q-Sepharose fractions, indicating very similar native molecular weight. The purity of the laccase is tested on SDS-PAGE.

4. Protein analysis

PAGE and native IEF are carried out on a Mini Protean II and a Model 111 Mini IEF cells(Bio-Rad). Western blots are carried out on a Mini trans-blot cell(Bio-Rad) with an alkaline phosphatase assay kit(Bio-Rad). The primary

antibodies are diluted 1000-fold during blotting. N-terminus sequencing is performed on an Applied Biosystems (ABI) 476A protein sequencer using liquid phase TFA delivery for cleavage and on-line HPLC for identification of PTH-amino acids. Standard Fast Cycles and Pre-Mix Buffer System is used according to manufacturer's instructions. Deglycosylation with glycosidase is done as follows: 3μg of protein and 3.6 units of glycosidase in 0.25M NaAc, pH 5, 20 mM EDTA, 0.05% 2-mercaptoethanol is incubated at 37°C for 18 hours with ovalbumin and bovine serum albumin serving as positive and negative control, respectively, and the mobility is detected by SDS-PAGE.

Amino acid analysis for determining extinction coefficients is done using Amino Quant 1090 HPLC system from Hewlett Packard. Microwave facilitated vapor phase hydrolysis of lyophilized samples is done using the MDS-2000 hydrolysis-station(CEM, Matthews, NC). 6N HCl containing 1% phenol as a scavenger is used to create the acid vapors. Hydrolysis time is 20 minutes at 70 psi (~148°C). Hydrolyzed samples are lyophilized and redissolved in 20 μl of 500pmol/μl sarcosine and norvaline as internal standards. 1μl is injected and analyzed according to manufacturer's instructions.

B. RESULTS AND DISCUSSION

1. Purification

The previously characterized *P. pinsitus* laccase has a pI of ~3.5. However, considerable laccase activity is detected in the run-through fraction of Q-Sepharose pre-equilibrated at pH 7.7. Upon a gradient elution, one more active fraction comes off the column before the active fraction initially anticipated. UV-visible spectra and SDS-PAGE show that all three fractions contain mainly laccase. After further purification by gel filtration, different pI's under native non-denaturing conditions are detected for the

two new fractions and shown to be consistent with the elution order.

2. Characterization

The pure laccase preparations derived from Q-Sepharose eluates behave as a rather well-defined band on SDS-PAGE at ~63 kDa. Deglycosylation detects ~14% w/w carbohydrates based on mobility change on SDS-PAGE. On native-IEF, the laccase preparations have bands of pI 6-6.5, 5-6.5, and 3.5. ABTS-agarose overlay show that all bands are active. Each form in turn shows multiple isoforms under the IEF conditions.

The neutral and acidic forms have a typical UV-visible spectrum with maxima at 605 and 275 nm. The ratio of A_{275}/A_{605} is 30-40. The spectrum for the acidic-neutral form has a peak at 276 nm and a shoulder around 600 nm.

The N-terminal sequencing shows that the neutral and neutral-acidic forms have the same first 29 residues(Table 1). The N-terminus of the acidic form matches 100% to that of the previously characterized form. All three forms exhibit comparable cross-reactivity toward antibodies raised against previously characterized form.

Table 1. Structural and enzymatic properties of *P. pinsitus* laccases

Form	N-terminus	LACU	ΔA_{405}min-1(ABTS)
Acidic	GIGPVA D LTITNAAVSPDGFSRQAVVVNG	92	4000
Acidic-Neutral	A*****(*)*VVA**P******L*D*I****	75	4000
Neutral	A*****(*)*VVA**P******L*D*I****	32	1000

*:Same residue as compared with the acidic form. (): weak signal

3. Laccase Activity

The specific activities(per A_{275}) of the three forms are tested by both ABTS and syringaldazine oxidations. The shapes and optima of the pH activity profiles for the three forms are very close: all have optima at ≤pH4 and pH 5-5.5 for ABTS and syringaldazine oxidations, respectively.

IV. ISOLATION OF MULTIPLE COPIES OF *POLYPORUS PINSITUS* LACCASE ENZYMES AND GENES

A. MATERIALS AND METHODS

1. Strains

The following strains are employed in the methods described below: *E. coli* K802(*e14-(mrca), mcrB, hsdR2, galK2, galT22, supE44, metB1*; Clonetech); *E. coli* XL-1 Blue(*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F'proAB,* lacIqZDM15, Tn10(tet^r)];Stratagene) and *Polyporus pinsitus* CBS 678.70.

2. Genomic DNA isolation

Cultures of *P.pinsitus* are grown in 500 ml YG (0.5% yeast extract, 2% dextrose) at room temperature for 3 to 4 days. Mycelia are harvested through miracloth, washed twice with TE and frozen quickly in liquid nitrogen. The frozen mycelia are stored at -80°C. To isolate DNA, the mycelia

are ground to a fine powder in an electric coffee grinder. The powdered mycelia are resuspended in TE to a final volume of 22 ml. Four ml 20% SDS is added with mixing by inversion followed by incubation at room temperature for 10 minutes. The sample is gently extracted with phenol:chloroform and centrifuged to separate the phases. The aqueous phase is collected and 400μl proteinase A(10 mg/ml stock) is added. The sample is incubated at 37°C for 30 minutes followed by a phenol:chloroform extraction. The aqueous phase is precipitated by the addition of 0.1 volumes of 3 M Na acetate, pH 5.2 and 2.5 volumes 95% ethanol and freezing at 20°C for one hour. After centrifugation to precipitate the DNA, the pellet is resuspended in 6 ml TE, and 200 μl boiled RNase A(10 mg.ml stock) is added. After incubation at 37°C, 100 μl proteinase A(10 mg/ml stock) is added followed by incubation at 37°C for 30 minutes. The sample is phenol:chloroform extracted twice. To the aqueous phase, 0.1 volumes 3 M Na acetate and 2.5 volumes are added, and teh sample is frozen at -20°C for 1 hour. Following centrifugation, the pellet is gently resuspended in 400 μl TE, and 40 μl Na acetate and 1 ml 95% ethanol are added. The DNA is pelleted by centrifugation, and the pellet is washed in 70% ethanol. The final pellet is resuspended in 250 μl TE.

3. RNA preparation

RNA is isolated from mycelia which are harvested from *P. pinisitus* cultures which are either induced for laccase expression by the addition of 2,5-xylidine or are uninduced. The mycelia are washed and frozen quickly in liquid N_2. Frozen mycelia are ground to a fine powder in an electric coffee grinder. The powder is immediately suspended in 20 ml extraction buffer (0.2 M Tris-HCl, 0.25 M NaCl, 50 mM EGTA, 0.8% tri-isopropyl naphthalene sulfonic acids, 4.8% p-aminosalicylic acid, pH 8.5). All solutions for RNA

extraction are made with diethylpyrocarbonate(DEP)-treated water. The sample is kept on ice and 0.5 volumes TE-saturated phenol:chloroform is added. The sample is mixed well by inversion for 2 minutes, and the phases are separated by centrifugation. The aqueous phase is saved, and the organic phase is extracted with 2 ml extraction buffer and incubated at 68°C for 5 minutes. After centrifugation to separate the phases, the aqueous phases are pooled and extracted several time with phenol:chloroform until there is no longer any protein at the interface. To the aqueous phase 0.1 volume 3 M Na-acetate, pH 5.2 and 2.5 volumes 95% ethanol are added to precipitate the RNA, and the sample is frozen at -20°C for 2 hours. The RNA is pelleted and resuspended in DEP water with RNase inhibitor.

4. DNA sequencing

Nucleotide sequences are determined using TAQ polymerase cycle sequencing with fluorescent-labeled nucleotides, and reactions are electrophoresed on an Applied Biosystems automatic DNA sequencer(Model 363A, version 1.2.0).

5. Preparation of genomic libraries

Two size-selected genomic libraries of *P. pinsitus* are constructed. A library of 5 to 6 kb BamHI fragments are constructed in pBluescript+. Genomic DNA is digested with BamHI, and the digest is electrophoresed on a preparative agarose(IBI) gel. The region containing the 5 to 6 BamHI fragments is sliced from the gel. The DNA is isolated from teh gel using a Geneclean kit(BIO 101). The DNA is ligated into pBluescript plasmid previously digested with BamHI and dephosphorylated with BAP(GIBCO BRL), *E. coli* XL-1 Blue competent cells (Stratagene) are transformed with the ligation, and 12,000 white colonies are obtained.

A library of 7 to 8 kb BamHI/EcoRI fragments is constructed in pUC118. Ten μg genomic DNA is digested with

BamHI and EcoRI and treated with BAP(GIBCO BRL). Competent *E. coli* XL-1 Blue cells are transformed with the ligation, and the library contains ~8000 recombinants.

For the preparation of a total genomic library in lambda EMBL4, 25 μg of *P. pinsitus* genomic DNA is partially digested with Sau3A. After digestion, the DNA is electrophoresed on a preparative low-melt agarose gel, and a band containing the 9 to 23 kb sized DNA is sliced from the gel. The DNA is extracted from the gel using ß-agarose(New England Biolabs). The isolated EMBL4 arms (Clonetech) according to the supplier's directions. The ligation is packaged *in vitro* using a Gigapack II kit(Stratagene). The library is titered using *E. coli* K802 cells. The unamplified library is estimated to contain 35,000 independent recombinants. The library is amplified using *E. coli* K802 cells.

6. Southern and Northern Blots

DNA samples are electrophoresed on agarose gels in TAE buffer using standard protocols. RNA samples are electrophoresed on agarose gels containing formaldehyde. Both DNA and RNA gels are transferred to Zeta-Probe membrane(BIO-RAD) using either capillary action under alkaline conditions or a vacuum blotter. After transfer, the DNA gels are UV crosslinked. Blots are prehybridized at 65°C in 1.5X SSPE, 1% SDS, 0.5% non-fat dried milk and 200 μg/ml salmon sperm DNA for 1 hour. Radioactive probes are added directly to the prehybridization solutions, and hybridizations are continued overnight at 65°C. Blots are washed with 2XSSC for 5 minutes at 65°C and with 0.2XSSC, 1%SDS,0.1% Na-pyrophosphate at 65°C for 30 minutes twice.

Radioactive labeled probes are prepared using a α-^{32}P-dCTP and a nick translation kit(GIBCO-BRL).

7. Library screening

For screening of the size-selected 5-6 kb BamHI and 7-8 kb BamHI/EcoRI libraries ~500 colonies on LB carb plates and lifted the colonies to Hybond N^+ filters(Amersham) using standard procedures. The filters are UV crosslinked following neutralization. The filters are prehybridized at 65°C in 1,5X SSPE, 1% SDS, 0.5% non-fat dried milk, 200 µg/ml salmon sperm DNA for 1 hour. Nick-translated probes are added directly to the prehybridization solution, and hybridizations are done overnight at 65°C.

For screening of the genomic bank in EMBL, appropriate dilutions of the amplified library are plated with *E. coli* K802 cells on 100mM NZY top agarose. The plaques are lifted to Hybond N^+ membranes(Amersham) using standard procedures. The DNA is crosslinked to the membranes using UV crosslinking. The filters are prehybridized and hybridized using the same conditions as those mentioned above.

RESULTS AND DISCUSSION

1.Isolation of multiple copies of laccase gene

P. pinsitus genomic DNA is digested with several different restriction enzymes for southern analysis. The blot is probed with the cDNA insert(isolated as a BamHI/SphI fragment from the pYES vector) which is labeled with α-P^{32}-dCTP. The blot is hybridized and washed as described above. The cDNA hybridizes to several restriction fragments for most of the enzymes suggesting that there are multiple laccase genes in the genome. Because the cDNA hybridizes to a BamHI fragment of ~5.5 kb, a library of 5-6 kb BamHI fragments from *P. pinisitus* is constructed.

2. Screening of Genomic Libraries

The results from screening of the libraries are summarized in Table 2. The 5-6 kb BamHI size-selected library is screened with the original cDNA clone labeled with ^{32}P. Approximately 30,000 colonies are screened with hybridizations done at 65°C. Plasmid DNA is isolated from

two positive colonies and digested with BamHI to check for insert size. Both clones contain an ~5.5 kb BamHI insert. The cloned insert(LCC3) is sequenced from either end; the sequence has homology to the cDNA, but is clearly not the cDNA encoded laccase. The partial DNA sequence of LCC3 also indicates that the LCC3 pUC118 clone does not contain the full gene.

From a southern blot of BamHI/EcoRI double digested DNA it is demonstrated that the cDNA hybridizes to an ~7.7 kb fragment. A size-selected library in pUC118 is constructed containing 7-8 BamHI/EcoRI fragments. A total of ~8000 independent colonies are obtained and screened by hybridization with a ^{32}P labeled insert. Plasmid DNA is isolated from the positive colonies and digested with BamHI and EcoRI. Restriction analysis of the plasmids demonstrate that they fall into two classes. One class (LCC4) contains four clones which are all identical and have an ~7.7 kb BamHI/EcoRI insert which hybridizes to the cDNA. A second class(LCC1) contains two clones which are identical and have inserts of ~7.2 kb which hybridize to the cDNA. Partial DNA sequencing of clones LCC1 and LCC4 demonstrate that clone 21 is the genomic clone of the original cDNA, while LCC4 codes for another laccase. The partial DNA sequence of LCC1 shows that the pUC118 clone does not contain the full gene and that a fragment upstream of the EcoRI site is needed.

At the same time the size selected 7-8 BamHI/EcoRI library is being constructed, a *P. pinisitus* genomic bank in EMBL4 is constructed containing ~35,000 independent recombinant phage. Ten positive plaques are picked and purified. DNA is isolated from the purified phage lysates. Restriction digests of EMBL DNAs demonstrates that there are three classes of clones. The first class(11GEN) is defined by two sibs whose inserts contain a BamHI/EcoRI fragment of the same size as LCC1 which hybridizes to the LCC1 insert.

The second class(12GEN) contains one clone which has a different restriction pattern than the 11GEN class and whose insert contains a different restriction pattern than the 11GEN class and whose insert contains an ~5.7 kb BamHI/EcoRI fragment. The third class is defined by a single clone whose insert contains an ~3.2 kb BamHI/EcoRI fragment which hybridizes to the LCC1 insert. DNA sequence analysis demonstrates that clone 11GEN contains the LCC1 BamHI/EcoRI fragment and both 5' and 3" flanking regions. It is also demonstrated that clone 12GEN contains a portion of the LCC1 insert.

The *P. pinisitus* EMBL genomic bank is also screened with the LCC3 BamHI insert in order to clone the full gene. Approximately 30,000 plaques are plated and lifted from hybridization. Five plaques which hybridize to the LCC3(BamHI/EcoRI) insert are identified and purified. DNA is isolated from the purified phage stocks. Southern analysis of *P. pinisitus* genomic DNA demonstrates that the LCC3 BAmHI insert hybridizes to an ~7kb EcoRI fragment. Restriction digests and southerns demonstrate that 4 of the clones contain restriction fragments which hybridize to the EcoRI/BamHI(1.6 kb) fragment and that the clones fall into three classes. Class one is defined by a single clone(LCC5) whose insert contains a 3kb EcoRI fragment which hybridizes to the LCC3 BamHI/EcoRI fragment. Another class is defined by clone(LCC2) whose insert contains an ~11 kb EcoRI fragment which hybridizes to the LCC3 BamHI/EcoRI insert. The third class is defined by two clones which are not identical but contain many of the same restriction fragments; these clones both contain an ~7.5 kb EcoRI fragment which hybridizes to the LCC3 insert. Further analysis of this third class indicates that they are identical to clone LCC4. Partial DNA sequencing of LCC5 and LCC2 indicates that both of these clones code for laccases;

however, neither is identical to any of the above mentioned laccase genes(LCC1, LCC3, or LCC4). At this point, five unique laccase genes are cloned; however, the fragments subcloned from LCC5 and LCC2 do not contain the full genes.

From the DNA sequencing of the 3 kb EcoRI fragment from clone LCC5 it is determined that ~200 base pairs of the N-terminus are upstream of the EcoRI site. A 380 bp EcoRI/MluI fragment from LCC5 is used to identify for subcloning a MluI fragment from the LCC5 EMBL clone. An ~4.5 MluI fragment from the LCC5 EMBL clone is subcloned for sequencing and shown to contain the N-terminal sequence.

To clone the N-terminal half of the LCC3 laccase gene, the *P. pinsitus* EMBL genomic bank is probed with an ~750 bp BamHI/StuI restriction fragment from the LCC3 pUC118 clone. Approximately 25,000 plaques are screened and five plaques appear to hybridize with the probe. Upon further purification only three of the clones are still positive. Two of the clones give very strong signals and the restrictions digests of DNA isolated from these phage demonstrate that both contain an ~750 bp BamHI/StuI fragment in their inserts and that the two clones are not identical but overlapped. Based on results of Southern analysis, an ~8.5 kb fragment from these clones are subcloned for sequencing. The EcoRI fragment is shown to contain the entire gene.

To clone the N-terminal half of the LCC2 laccase gene, the *P. pinsitus* genomic bank in EMBL4 is probed with an ~680 bp EcoRI/PvuI of the EMBL LCC2 clone. Thirty thousand plaques are screened by hybridization at 65°C, and 15 plaques appear to hybridize with the probe. All fifteen are purified, and DNA is isolated. The clones can be placed in four classes based on restriction patterns, Seven of the clones are all sibs, and are identical to the original EMBL clone of LCC2. The second class is defined by 3 clones

which are sibs. An ~4 kb HindIII fragment is subcloned from this class for sequencing and is shown to contain the N-terminal half of LCC2. A third class is defined by a single clone and is not characterized further.

3. DNA sequencing

The complete DNA sequences of the five genomic clones is determined as described in Materials and Methods. Sequencing of clone LCC2 demonstrate that it probably codes for the second form of laccase(neutral pI) isolated from culture broth from an induced *P. pinsitus* culture as described above. The N-terminal protein sequence from the neutral pI laccase and the predicted N-terminus for the protein coded for by LCC2 are compared, and show identity. The predicted pI for the protein coded for by clone LCC2 is 5.95, which is in good agreement with the experimental pI determined for the second form of laccase being between 5.0 and 6.5. Figures 1-5 (SEQ ID NOS. 1-5) show the DNA sequences and predicted translation products for the genomic clones. For LCC1, the N-terminus of the mature protein as determined by protein sequencing and predicted by Von Heijne rules is Gly at position 22. The N-terminus is Gly-Ile-Gly-Pro-Val-Ala-. For LCC2 the N-terminal amino acid of the mature protein as determined by protein sequencing is Ala at position 21. The N-terminus is Ala-Ile-Gly-Pro-Val-Ala-. For LCC3 the predicted N-terminal amino acid of the mature protein is Ser at position 22, with the N terminus being Ser-Ile-Gly-Pro-Val-Thr-Glu-Leu-. For LCC4, the predicted N-terminal amino acid is Ala at position 23 with the N-terminus being Ala-Ile-Gly-Pro-Val-Thr-. For LCC5 the predicted N-terminal amino acid is Ala at position 24 with the N-terminus being Ala-Ile-Gly-Pro-Val-Thr-Asp. A comparison of the structural organization of the genes and the predicted proteins they code for is presented in Table 1. It will be seen that the five genes have different

structural organizations and code for proteins of slightly different sizes. Comparisons between the predicted proteins of the genomic clones and other fungal laccase are also done. Table 2 shows a comparison of the predicted laccase to each other and to other fungal laccases. Clone LCC1(the induced laccase first characterized) has the most identity(90%) to the *Coriolus hirsutus* laccase and the PM1 basidiomycete laccase(Coll et al., *supra*). The other four laccases have between 64 and 80% identity to the *C. hirsutus* laccase. The laccase coded for by LCC3 has the least identity to the LCC1 laccase and the other fungal laccases shown in Table 2. LCC2 appears to be the second wild-type laccase isolated as described above; based on the N-terminal sequences of the isolated clones, it also appears that the "neutral" and acidic neutral" wild-type laccases are the same enzyme which is encoded by the LCC2 sequence.

Table 1 Comparison of Structural Organization and Predicted Proteins of the *P. pinsitis* Genomic Clones.

Gene	# Introns	Size of Predicted Precursor Protein	Size of Predicted Mature Protein	Predicted Isolelectric Point
21GEN	8	520	499	4.49
23GEN	10	519	498	5.95
24GEN	12	516	495	5.23
31GEN	11	510	488	4.06
41GEN	11	527	504	4.07

Table 2 Amino Acid Identity Between *P. pinsitis* Laccases and Other Fungal Laccases.

	21GEN	23GEN	24GEN	31GEN	41GEN	CRIPHA	CRIPHE	PBILAC	PM1
21GEN	———	79%	64%	70%	72%	90%	91%	64%	80%
23GEN	79%	———	65%	66%	69%	80%	81%	62%	74%
24GEN	64%	65%	———	61%	65%	64%	65%	61%	63%
31GEN	70%	66%	61%	———	75%	69%	70%	64%	69%
41GEN	72%	69%	65%	75%	———	71%	72%	64%	71%
CRIPHA	90%	80%	64%	69%	71%	———	99%	64%	80%
CRIPHE	91%	81%	65%	70%	72%	99%	———	65%	81%
PBILAC	64%	62%	61%	64%	64%	64%	65%	———	65%
PM1	80%	74%	63%	69%	71%	80%	81%	65%	———

21GEN, 23GEN, 24GEN, 31GEN and 41GEN= *P. pinsitis* laccase clones

CRIPHA= *Coriolus hirsutis* laccase A

CRIPHE= *C. hirsutis* laccase B

PBILAC= *Phlebia radiata* laccase

PM1= Basidiomycete PM1 laccase (CECT2971)

5. Northern blots

RNA is isolated from mycelia from both a xylidine-induced culture and an uninduced culture. RNA is blotted to membrane after electrophoresis, and the blot is probed with the cDNA insert, or a small fragment containing ~100 bp of the 23GEN promoter and the first 100 bp of the coding region. A transcript of about 1.8 kb hybridizes to both the induced and uninduced RNA samples; however, transcription of this message is clearly induced by the addition of xylidine to the culture.

III. EXPRESSION OF *P. PINSITUS* LACCASE IN *ASPERGILLUS*

MATERIALS AND METHODS

1. Strains

A. oryzae A1560, *A. oryzae* HowB104(fungamyl delete, *pyrg*), *A. oryzae* HowB101*pyrg*, *A. niger* Bo-1, *A. niger* Bo-80, *A. niger* ATCC1040, *A. niger* NRRL337, *A. niger* NRRL326, *A. niger* NRRL326, *A. niger* NRRL2295, *A. niger* ATCC11358, *A. niger* NRRL322, *A. niger* AT10864, *A. japonicus* A1438, *A. phoenicis*, *A. foetidus* N953.

2. Media

For the shake flask cultivation of the *A. niger, A. foetidus,* and *A. phoenicis* MY50 (per liter:50 g maltodextrin, 2 g $MgSO_4 \cdot H_2O$, 10 gKH_2PO_4, 2 g K_2SO_4, 2 g citric acid, 10 g yeast extract, 0.5 ml trace metals, 2 g urea, pH 6.0) media is used. For the shake flask cultivation of the *A. oryzae* A1560 and HowB101 strains MY51(per liter: 30 g maltodextrin, 2 mg $MgSO_4$. 10 g KH_2PO_4, 2 g K_2SO_4, 2 g citric acid, 10 g yeast extract, 0.5 ml trace metals, 1 g urea, 2 g$(NH_4)_2SO_4$, pH 6.0) is used. For the shake flask analysis of the *A.oryzae* HowB104 strains, MY51 maltose(same as MY51 but with 50g of maltose instead of maltodextrin) media is used. For the shake flask analysis of the *A. japonicus* strains M400 media(per liter: 50 g maltodextrin, 2 g $MgSO_4$, 2 g

KH_2PO_4, 4 g citric acid, 8 g yeast extract, 0.5 ml trace metals, 2 g urea, pH 6.0.

Cultures grown overnight for protoplast formation and subsequent transformation are grown in YEG(0.5% yeast extract, 2% dextrose). For strains that are *pyrg*, uridine is supplemented to 10 mM final concentration.

3. Screening for laccase production

Primary transformants are screened first on a minimal medium plates containing 1% glucose as the carbon source and 1mM ABTS to test for production of laccase. Transformants that give green zones on the plates are picked and spore purified before shake flask analysis is done.

Shake flask samples are centrifuged to clear the broth. Dilute or undiluted broth samples are assayed with ABTS

RESULTS AND DISCUSSION

1. Expression in shake flasks

The first expression vector constructed is pDSY1, which contains the TAKA promoter, TAKA signal sequence, *P. pinisitus* laccase cDNA beginning at the mature N-terminus and the AMG terminator. The TAKA signal sequence: laccase insert is constructed in 2 steps. First by site directed mutagenesis, an AgeI site beginning at bp 107 of the laccase mature coding region is created by a single base change and a NsiI site is created ~120 bp downstream of the laccase stop codon(ACG GGT->ACC GGT and TTC GCT->ATG CAT, respectively). A small PCR fragment beginning with an SfiI site and ending with the AgeI site at 107 bp in laccase is PCR amplified. This fragment contains a piece of the TAKA signal sequence and the first ~107 bp of the mature laccase cDNA. Further DNA sequencing of this fragment shows it has a single base change that leads to a substitution of Asn for Thr at position 9 in mature laccase. This substitution creates a potential N-linked glycosylation site. The PCR

fragment and AgeI/NsiI fragments are cloned into pMWR1(Figure 6) which has been digested with SfiI/NsiI. The vector pMWR1 contains the TAKA promoter, a portion of the TAKA signal sequence which ends with an SfiI site, and the TAKA terminator with a NsiI site inserted directly 5' to the terminator. The resulting expression vector (Figure 7) is used to cotransform several hosts. Methods for co-transformation of *Aspergillus* strains are as described in Christensen et al., *supra*.

In the second laccase expression vector, the base change in DSY1 which leads to the substitution of Asn for Thr at amino acid 9 is reverted back to wild type by a PCR reaction. The second expression vector pDSY2 is identical to pDSY1 except for this single base change. Three different *A. oryzae* strains and several *A. niger* strains are cotransformed with pDSY2 and either pTOC90(WO 91/17243) which carries the *A. nidulans amdS* gene or pSO2 which carries the *A. oryzae pyrG* gene.

Expression of laccase is observed in all hosts tested, with both DSY1 and DSY2. Yields range from 0.1-12.0 Δabs/min/ml, with highest yields being observed with *A. niger* strains.

A construct pDSY10 is made which contains the TAKA promoter, laccase full-length cDNA including its own signal sequence and the AMG terminator. A 200 bp BamHI/AgeI fragment which has a BamHI site immediately 5' to the ATG of the initiation codon and an AgeI site at the same position as in pDSY1 is PCR amplified using *lac*1 as template. A MluI/HindIII fragment is PCR amplified using pDSY2 as template and begins with the MluI site present in the cDNA and ends with a HindII site directly 3' to the stop codon of laccase. The above two fragments and the AgeI/MluI fragment

from pDSY2 are ligated into pHD414 to yield pDSY10(Figure 8).

The vector pHD414 used in expression of laccase is a derivative of the plasmid p775(EP 238 023). In contrast to this plasmid, pHD414 has a string of unique restriction sites between the TAKA promoter and the AMG terminator. The plasmid is constructed by removal of an approximately 200 bp long fragment (containing undesirable RE sites) at the 3' end of the terminator, and subsequent removal of an approximately 250 bp long fragment at the 5' end of the promoter, also containing undesirable sites. The 200 bp region is removed by cleavage with NarI (positioned in the pUC vector) and XbaI (just 3' to the terminator), subsequent filling in the generated ends with Klenow DNA polymerase + dNTP, purification of the vector fragment on a gel and religation of the vector fragment. This plasmid is called pHD413. pHD413 is cut with StuI (positioned in the 5' end of the promoter) and PvuII (in the pUC vector), fractionated on gel and religated, resulting in pHD414. Cotransformation of *A. oryzae* HowB104 and *A. niger* Bo-1 are done using pToC90 for selection. Yields in shake flask are comparable to those seen with pDSY2.

2. Expression in fermentors

A 1 ml aliquot of a spore suspension of *Aspergillus niger* transformant Bo-1-pDSY10-4(approximately 10^9 spores/ml) is added aseptically to a 500 ml shake flask containing 100 ml of sterile shake flask medium (glucose, 75g/l; soya meal, 20 g/l; $MgSO_4 \cdot 7H_2O$, 2g/l; KH_2PO_4, 10g/l; K_2SO_4, 2g/l; $CaCl_2 \cdot 2H_2O$ 0.5 g/l; Citric acid, 2g/l; yeast extract, 10g/l; trace metals[$ZnSO_4 \cdot 7H_2O$, 14.3 g/l; $CuSO_4 \cdot 5H_2O$, 2.5 g/l; $NiCl_2 \cdot 6H_2O$, 0.5 g/l; $FeSO_4 \cdot 7H_2O$, 13.8 g/l, $MnSO_4 \cdot H_2O$, 8.5 g/l; citric acid, 3.0 g/l], 0.5 ml/l; urea, 2g/l, made with tap water and adjusted to pH 6.0 before autoclaving), and incubated at 37°C on a rotary shaker at 200 rpm for 18

hours. 50 ml of this culture is aseptically transferred to a 3 liter fermentor containing 1.8 liters of the fermentor media (maltodextrin MD01 300 g/l; $MgSO_4 \cdot 7H_2O$, 2g/l; KH_2PO_4, 2g/l; citric acid 2g/l; K_2SO_4, 2.7 g/l;$CaCl_2 \cdot 2H_2O$, 2g/l; trace metals, 0.5 ml/l; pluronic antifoam, 1ml/l; made with tap water and pH adjusted to 6.0 before autoclaving). The fermentor temperature is maintained at 34°C by the circulation of cooling water through the fermentor jacket. Sterile air is sparged through the fermentor at a rate of 1.8 liter/min (1v/v/m). The agitation rate is maintained at 800 rpm for the first 24 hours after inoculation and at 1300 rpm for the remainder of the fermentation. The pH of the fermentation is kept at 4.0 by the automatic addition of 5N NaOH or H_3PO_4. Sterile feed (urea, 50 g/l; pluronic antifoam, 1.5 ml/l, made up with distilled water and autoclaved) is added to the fermentor by use of a peristaltic pump. The feed rate profile during the fermentation is as follows: 40 g of feed is added initially before inoculation; after inoculation, feed is at a constant rate of 2.5 g/l h.

Copper is made as a 400X stock in water or a suitable buffer, filter sterilized and added aseptically to the tank to a final level of 0.5 mM. Samples for enzyme activity determination are withdrawn and filtered through Miracloth to remove mycelia. These samples are assayed for laccase activity by a LACU assay. Laccase activity is found to increase continuously during the course of the fermentation, with a value of approximately 55 LACU/ml is achieved after 190 hours. This corresponds to approximately 350mg/l of recombinant laccase expressed.

IV. PURIFICATION OF RECOMBINANT LACCASE

MATERIALS AND METHODS

1. Materials.

Chemicals used as buffers and substrates are commercial products of at least reagent grade. Endo/N-glycosidase G is

from Boehringer-Mannheim. Chromatography is performed on either a Pharmacia's FPLC or a conventional open column low pressure system. Spectroscopic assays are conducted on a Shimadzu PC160 spectrophotometer.

2. Purification

(a) DSY2

2.8 liters cheese-cloth filtered broth(pH 7, 19mS) obtained from an A. oryzae pDSY2 transformant as described above is filtered on 0.45 μ Corning filter and concentrated on Spiral Concentrator(Amicon) with S1Y30 membrane to 200ml. The concentrate pH is adjusted to 7.5, diluted with 4.8 l water to achieve 1.2 mS, and concentrated on S1Y30 to 200ml. 50ml of this broth solution is applied onto a Q-Sepharose column(XK16, 34ml gel), pre-equilibrated with 10mM Tris, pH 7.5, 0.7 mS(Buffer A). The blue laccase band that migrates slowly during loading is eluted by a linear gradient of Buffer B(Buffer A plus 0.5 M NaCl). 24 ml of pooled laccase fractions are concentrated on Centricon-100(Amicon) to 4.5 ml and applied onto a Superdex 200 column(HiLoad 16/60, 120 ml gel). During the development with Buffer C(Buffer A plus 0.15 M NaCl, 14.4 mS), the blue laccase fractions elute followed by brownish contaminant fractions. Only the first half of the elution band(detected by Abs_{600}) show a high laccase to contaminant ratio and are pooled. The pooled fractions are dialyzed in 10mM Bis-Tris, pH 6.8, 0.6mS(Buffer D), applied onto a Mono-Q column(Mono-Q 5/5, 1ml) equilibrated with Buffer D, and eluted with Buffer E(Bufer D plus 0.5 M NaCl) using a linear gradient. The laccase fractions, which ome out round 27% Buffer E, are pure as judged by SDS-PAGE. At each step, the laccase fractions are routinely checked by ABTS oxidation, SDS-PAGE, and Western Blot.

(b) DSY10

2.8 liters cheese-cloth filtered broth(pH 7.3, 24mS) obtained from HowB104-pDSY10 is filtered on Whatman #2 paper and concentrated on Spiral Concentrator(Amicon) with S1Y100 membrane to 210ml. The concentrate pH is diluted with water to achieve 1.2 mS, and concentrated on S1Y100 to 328 ml. This broth solution is applied onto a Q-Sepharose column(XK26, 120 ml gel), pre-equilibrated with 10mM Tris, pH 7.5, 0.7 mS(Buffer A). The blue laccase band that migrates slowly during loading is eluted by a linear gradient of Buffer B(Buffer A plus 2 M NaCl). 120 ml of pooled laccase fractions are diluted with water to achieve 1.1mS and then concentrated on SIY100 to 294 ml and applied onto a Mono-Q column(HiLoad 16/10, 40 ml gel) pre-equilibrated with Buffer A. The laccase slowly passes through the column during loading and washing with Buffer A. The pooled fractions which have a pH reading of 5.6, are loaded on a Mono-Q column(HiLoad 16/10, 40 ml gel), pre-equilibrated with Buffer C(10mM MES, pH 5.5, 0.1 mS). The laccase fractions elute by a very shallow gradient of Buffer D(Buffer C + 1M NaCl). Enzymatic assays are conducted as described above.

3. Protein analysis

Total amino acid analysis, N-terminal sequencing, deglycosylation, SDS-PAGE, IEF, and Western blots are performed as decribed above.

B. RESULTS AND DISCUSSION

1. Purification and Characterization

Overall a 256-fold purification and a yield of 37% are achieved for DSY10, and a 246-fold purification and a yield of 14% are achieved for DSY2 In terms of electorphoretic pattern, spectral properties and activity, purified DSY2 and DSY10 are indistinguishable. Purified recombinant laccases behave as a dimer on gel filtration, and exhibit subunit molecular weight which is somewhat larger than that of the

wild type laccase, indicating a post-translational processing in *A. oryzae* that results in the extra glycosylation on the recombinants. Deglycosylation has confirmed the difference in mass arising from extra sugars(Table 3).

Table 3. Molecular and spectral properties of recombinant and wild-type laccase

	MW, kDa Native	subunit	Carbohydrate w/w%	pI	λ_{max}, nm (ε, $1/g*cm$)
WT	~130	~63	~7	3.5	275(1.8)615(0.12)
Rec.	~130	~67	~13	3.5	275(1.7)615(0.11)

The spectra of the purified laccases have maxima of 615 nm and 275, with the ratio of absorbance at 275 nm to that at 615 nm being 16, indicating one Type I Cu per subunit. The ratio of absorbance at 330nm to that at 615nm is 1.0, close to the 0.75 value of *Rhus vernicefera* laccase, suggesting the presence of one Type II and two Type III copper ions per subunit. The extinction coefficient determined by amino acid analysis is 1.71/(g*cm),

3. Activity

The laccase activity is measured by syringaldazine and ABTS oxidations. Expressed per A_{275}, the laccase has a value of 83 for LACU. Expressed per mg, it has a LACU of 141. The pH profile of the laccase is provided in Figure 9.

V. USE OF *POLYPORUS* LACCASE TO DYE HAIR

The dyeing effect of *Polyporus pinsitus* laccase is tested and compared to the dyeing effect of 3% H_2O_2 on various dye precursors (listed below) and further on 0.1% p-phenylenediamine compared with a number of modifiers.

Materials:

Dye precursors:

0.1 % p-phenylene-diamine in 0.1 M K-phosphate buffer, pH 7.0. (pPD)

0.1 % p-toluylene-diamine in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % chloro-p-phenylenediamine in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % p-aminophenol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % o-aminophenol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % 3,4-diaminotoluene in 0.1 M K-phosphate, buffer pH 7.0.

<u>Modifiers:</u>

0.1 % m-phenylene-diamine in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % 2,4-diaminoanisole in 0,1 M K-phosphate buffer, pH 7.0.

0.1 % α-naphthol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % hydroquinone in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % pyrocatechol in 0.1 M K-phosphate buffer, pH 7.0.

0.1% resorcinol in 0.1 M K-phosphate buffer, pH 7.0.

0.1 % 4-chlororesorcinol in 0.1 M K-phosphate buffer, pH 7.0.

When a modifier is used, the dye precursor p-phenylene-diamine is combined with one of the above indicated modifiers so that the final concentration in the dyeing solution is 0.1 % with respect to precursor and 0.1 % with respect to modifier. The enzyme used is a recombinant laccase from *Polyporus pinisitus*, at a concentration of 10 LACU/ml.

Other solutions used in the process are 3% H_2O_2 (in the final dye solution), and a commercial shampoo.

The quantitative color of the hair tresses is determined on a Datacolor Textflash 2000 (CIE-Lab) by the use of

CIE-Lab parameters L* ("0"=black and "100"=white) combined with a* ("-"=green and "+"=red). DL* and Da* are the delta values of L* and a*, respectively, of a sample when compared to L* and a* of untreated hair. The Light fastness is determined under a day light bulb (D65) at 1000 LUX.

Hair tresses of blond European hair (1 gram) are used. 4 ml dye precursor solution (including modifier)is mixed with 1 ml laccase or 1 ml H_2O_2 on a Whirley mixer, applied to the hair tresses and kept at 30°C for 60 minutes. The hair tresses are then rinsed with running water, combed, and air dried.

The results of the dyeing effect test are displayed below in Table 4-6 and further in the graphs in Figures 10 to 12.

Table 4

Sample no.	Sample ID	L*	a*	DL*	Da*
	Untreated blond hair	72.25	2.42		
1	p-phenylenediamine (Reference)	62.85	4.03	-9.41	1,61
2	p-phenylenediamine + Laccase	28.70	0.33	-43.56	-2,10
3	p-phenylenediamine + 3% H_2O_2	21.88	2.04	-50.37	-0,39
4	p-Toluylenediamine (Reference)	58.14	4.34	-14.11	1.92
5	p-Toluylenediamine + Laccase	36.70	8.09	-35.56	5.67
6	p-Toluylenediamine + 3% H_2O_2	42.30	6.24	-29.95	3.81
7	chloro-p-phenylenediamine (Reference)	69.82	3.23	-2.43	0.81
8	chloro-p-phenylenediamine + Laccase	35.58	9.36	-36.68	6.93
9	chloro-p-phenylenediamine + 3% H_2O_2	45.42	9.59	-26.84	7.17
10	p-aminophenol (Reference)	66.62	5.03	-5.63	2.61
11	p-aminophenol + Laccase	42,42	7.38	-29,84	4.95
12	p-aminophenol + 3% H_2O_2	50.54	9.42	-21.72	7.26
13	o-aminophenol (Reference)	69.39	4.82	-2.89	2.39
14	o-aminophenol + Laccase	60.20	12.92	-12.05	10.50
15	o-aminophenol + 3% H_2O_2	63.49	10.38	-8.77	7.96
16	3,4-diaminotoluene (Reference)	69.62	3.57	-2.63	1.15
17	3,4-diaminotoluene + Laccase	39.51	3.15	-32.74	0.73
18	3,4-diaminotoluene + 3% H_2O_2	59.32	4.16	-12.94	1.74

L*: 0=black, 100=white   a*: -=green, +=red

Table 5

Sample no.	Sample ID	L*	a*	DL*	Da*
	Untreated blond hair	72.25	2.42		
19	p-phenylenediamine+ m-phenylenediamin (Reference)	58.82	0.43	-13,44	-1,99
20	p-phenylenediamine + m-phenylenediamin + Laccase	27.20	0.83	-45,05	-1,59
21	p-phenylenediamine + m-phenylenediamine + 3% H2O2	16.96	0.13	-55,29	-2,59
22	p-phenylenediamine + 2,4 - diaminoanisole (Reference)	35.37	-0.02	-36,89	-2,45
23	p-phenylenediamine + 2,4 - diaminoanisole + Laccase	24.56	2.99	-47,70	0,57
24	p-phenylenediamine + 2,4-diaminoanisole + 3% H2O2	15.06	2.21	-57,20	-0,21
25	p-phenylenediamine + α-naphthol (Reference)	54.33	2.54	-17,93	0,12
26	p-phenylenediamine + α-naphthol + Laccase	29.53	4.03	-42,72	1,60
27	p-phenylenediamine + α-naphthol + 3% H2O2	19.58	3.90	-52,68	1,47
28	p-phenylenediamine + hydroquinone (Reference)	53.25	4.08	-19,01	1,65
29	p-phenylenediamine + hydroquinone + Laccase	40.48	5.00	-31,77	2,58
30	p-phenylenediamine + hydroquinone + 3% H2O2	29.06	4.96	-43,20	2,53

L*: 0=black, 100=white   a*: -=green, +=red

Table 6

Sample no.	Sample ID	L*	a*	DL*	Da*
	Untreated blond hair	72.25	2.42		
31	p-phenylenediamine + pyrocatechol (Reference)	53.78	1.68	-18.47	-0.74
32	p-phenylenediamine + pyrocatechol + Laccase	30.77	2.64	-41.49	0.22
33	p-phenylenediamine + pyrocatechol + 3% H_2O_2	22.15	3.30	-50.11	0.88
34	p-phenylenediamine + resorcinol (Reference)	62.12	4.23	-10.14	1.81
35	p-phenylenediamine + resorcinol + Laccase	36.14	2.91	-36.11	0.49
36	p-phenylenediamine + resorcinol + 3% H_2O_2	23.94	3.16	-48.31	0.74
40	p-phenylenediamine + 4-chlororesorcinol (Reference)	61.18	4.70	-11.07	2.28
41	p-phenylenediamine + 4-chlororesorcinol + Laccase	36.00	2.76	-36.26	0.34
42	p-phenylenediamine + 4-chlororesorcinol + 3% H_2O_2	22.63	2.60	-49.63	0.18

L*: 0=black, 100=white   a*: -=green, +=red

The oxidative hair dyeing is carried out as described above, except that 50 LACU/ml *Polyporus pinsitus* laccase was used.

To test wash stability, the dyed hair tresses are wetted and washed for 15 seconds with 50 µl of commercial shampoo, and rinsed with water for 1 minute. The hair tresses are washed up to 20 times.

The results of the hair wash test are displayed in figure 13. It can be seen in figure 13 that the wash stability of hair washed up to 20 times is excellent, when using *Polyporus pinsitus* laccase for oxidative dyeing.

To test light fastness, tresses of blond european hair are used for testing the light fastness of hair dyed using *Polyporus pinsitus* laccase in comparison to hair dyed using H_2O_2. p-phenylene-diamine is the dye precursor. The dyeing of the hair is carried out as described above. One hair tress is kept dark, while an other is kept at day light (i.e. under a day light bulb (D65)), at approximately 1000 LUX) for up to 275 hours. The CIE-Lab-values are determined immediately after the dyeing of the hair, and further during exposure to day light.

The results of the test are displayed in figure 14. Figure 14 shows that the hair dyed with p-phenylene-diamine using *Polyporus pinsitus* laccase has the same light fastness as hair dyed using H_2O_2.

Deposit of Biological Materials

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria,

Illinois, 61604 on May 25, 1994 and given the following accession numbers.

Deposit	Accession Number
E. coli DH5α containing pDSY22(41GEN; an ~3.0 kb EcoRI insert)	NRRL B-21263
E. coli DH5α containing pDSY23(41GEN; an ~4.5 kb MluI insert; insert contains a small portion of the EcoRI fragment of pDSY22 and sequences 5' to the EcoRI fragment)	NRRL B-21268
E. coli XL-1 Blue containing pDSY21(31GEN; an ~7.7 kb EcoRI/BamHI insert)	NRRL B-21264
E. coli XL-1 Blue containing pDSY18(21GEN; an ~8.0 kb BamHI insert)	NRRL B-21265
E. coli DH5α containing pDSY19(23GEN; an ~4 kb HindIII insert)	NRRL B-21266
E. coli DH5α containing pDSY20(24GEN; an ~8.5 kb EcoRI insert)	NRRL B-21267

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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(B) STREET: 1445 Drew Avenue
(C) CITY: Davis, California
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(G) TELEFAX: (916) 758-0317

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(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsværd
(D) COUNTRY: Denmark
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(F) TELEPHONE: +45 4444 8888
(G) TELEFAX: +45 4449 3256
(F) TELEX: 37304

(ii) TITLE OF INVENTION: PURIFIED POLYPORUS LACCASES AND NUCLEIC ACIDS ENCODING SAME

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Novo Nordisk of North America, Inc.
(B) STREET: 405 Lexington Avenue, Suite 6400
(C) CITY and STATE: New York, New York
(D) COUNTRY: U.S.A.
(E) ZIP: 10174-6401

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: to be assigned
(B) FILING DATE: 15-June-1995

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/265,534
(B) FILING DATE: 24-June-1994

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Lowney, Karen A.
(B) REGISTRATION NUMBER: 31,274
(C) REFERENCE/DOCKET NUMBER: 4185.204-WO

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212 867 0123
(B) TELEFAX: 212 878 9655

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2418 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Polyporus pinsitus

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 414..464

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 534..589

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 710..764

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 879..934

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1001..1050

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1147..1197

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1354..1410

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1609..1662

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join (413..465, 533..590, 709..765, 878..935, 1000..1051, 1146..1198, 1353..1411, 1608..1663)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GCTTTTGCGA GATCCTCCAC ATACCACTCA CTACTTTCAA GTTCTTCAAC ATG TCG AGG   239
                                                       Met Ser Arg
                                                       1

TTT CAC TCT CTT CTC GCT TTC GTC GTT GCT TCC CTT ACG GCT GTG GCC      287
Phe His Ser Leu Leu Ala Phe Val Val Ala Ser Leu Thr Ala Val Ala
    5                   10                  15

CAC GCT GGT ATC GGT CCC GTC GCC GAC CTA ACC ATC ACC AAC GCA GCG      335
His Ala Gly Ile Gly Pro Val Ala Asp Leu Thr Ile Thr Asn Ala Ala
20                  25                  30                  35

GTC AGC CCC GAC GGG TTT TCT CGC CAG GCC GTC GTC GTG AAC GGC GGC      383
Val Ser Pro Asp Gly Phe Ser Arg Gln Ala Val Val Val Asn Gly Gly
                35                  40                  45

ACC CCT GGC CCT CTC ATC ACG GGT AAC ATG GTTCGTCTCG GCTCGCACTA        433
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            50                  55

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                70                  75

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                                                       80

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    85                  90                  95

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Gln Cys Pro Ile Ser Ser Gly His Ser Phe Leu Tyr Asp Phe Gln Val
100                 105                 110                 115

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Pro Asp Gln Ala

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                 Gly Thr Phe Trp Tyr His Ser His Leu Ser Thr Gln
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Tyr Cys Asp Gly Leu Arg Gly Pro Phe Val Val Tyr Asp Pro Asn Asp
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Pro Ala Ala Asp Leu Tyr Asp Val Asp Asn
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                                        Asp Asp Thr Val Ile
                                                160

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Thr Leu Val Asp Trp Tyr His Val Ala Ala Lys Leu Gly Pro Ala Phe
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Pro                                                        Leu
                                                           180

GGC GCC GAC GCC ACC CTC ATC AAC GGT AAG GGA CGC TCC CCC AGC ACG         1101
Gly Ala Asp Ala Thr Leu Ile Asn Gly Lys Gly Arg Ser Pro Ser Thr
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ACC ACC GCG GAC CTC TCA GTT ATC AGC GTC ACC CCG GGT AAA CGC             1146
Thr Thr Ala Asp Leu Ser Val Ile Ser Val Thr Pro Gly Lys Arg
            200                 205                 210

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Tyr Arg Phe Arg Leu Val Ser Leu Ser Cys Asp Pro Asn Tyr Thr Phe
            215                 220                 225

AGC ATC GAT GGT CAC AAC ATG ACG ATC ATC GAG ACC GAC TCA ATC AAC         1293
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Thr Ala Pro Leu Val Val Asp Ser Ile Gln Ile Phe Ala Ala Gln Arg
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Tyr Ser Phe Val
260

ATCGTATGGT CATGTAG CTC GAG GCC AAC CAG GCC GTC GAC AAC TAC TGG 1443
Leu Glu Ala Asn Gln Ala Val Asp Asn Tyr Trp
265 270

ATT CGC GCC AAC CCG AAC TTC GGT AAC GTC GGG TTC ACC GGC GGC ATT 1491
Ile Arg Ala Asn Pro Asn Phe Gly Asn Val Gly Phe Thr Gly Gly Ile
275 280 285 290

AAC TCG GCT ATC CTC CGC TAC GAT GGT GCC GCT GCC GTG GAG CCC ACC 1539
Asn Ser Ala Ile Leu Arg Tyr Asp Gly Ala Ala Ala Val Glu Pro Thr
295 300 305

ACA ACG CAA ACC ACG TCG ACT GCG CCG CTC AAC GAG GTC AAC CTG CAC 1587
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Pro Leu Val Thr Thr Ala Val
325

TGTTGCTGAC CTCGACCCCC ACAG CCT GGC TCG CCC GTC GCT GGT GGT GTC 1689
Pro Gly Ser Pro Val Ala Gly Gly Val
330 335

GAC CTG GCC ATC AAC ATG GCG TTC AAC TTC AAC GGC ACC AAC TTC TTC 1737
Asp Leu Ala Ile Asn Met Ala Phe Asn Phe Asn Gly Thr Asn Phe Phe
340 345 350

ATC AAC GGC ACG TCT TTC ACG CCC CCG ACC GTG CCT GTC CTG CTC CAG 1785
Ile Asn Gly Thr Ser Phe Thr Pro Pro Thr Val Pro Val Leu Leu Gln
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GTC TAC TCG CTT CCC TCG AAC GCC GAC ATC GAG ATC TCC TTC CCC GCC 1881
Val Tyr Ser Leu Pro Ser Asn Ala Asp Ile Glu Ile Ser Phe Pro Ala
390 395 400

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Thr Ala Ala Ala Pro Gly Ala Pro His Pro Phe His Leu His Gly His
405 410 415

GCG TTC GCG GTC GTC CGC AGC GCC GGC AGC ACG GTT TAC AAC TAC GAC 1977
Ala Phe Ala Val Val Arg Ser Ala Gly Ser Thr Val Tyr Asn Tyr Asp
420 425 430

AAC CCC ATC TTC CGC GAC GTC GTC AGC ACG GGG ACG CCT GCG GCC GGT 2025
Asn Pro Ile Phe Arg Asp Val Val Ser Thr Gly Thr Pro Ala Ala Gly
435 440 445 450

GAC AAC GTC ACC ATC CGC TTC CGC ACC GAC AAC CCC GGC CCG TGG TTC 2073
Asp Asn Val Thr Ile Arg Phe Arg Thr Asp Asn Pro Gly Pro Trp Phe
455 460 465

CTC CAC TGC CAC ATC GAC TTC CAC CTC GAG GCC GGC TTC GCC GTC GTG 2121
Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Val Val
470 475 480

TTC GCG GAG GAC ATC CCC GAC GTC GCG TCG GCG AAC CCC GTC CCC CAG 2169
Phe Ala Glu Asp Ile Pro Asp Val Ala Ser Ala Asn Pro Val Pro Gln
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Ala Trp Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Pro Ser Asp
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Gln
515

TGCAATACGG ACTCTCGCCT CATTATGGTT ACACACTCGC TCTGGATCTC TCGCCTGTCG 2330

ACAGAACAAA CTTGTATAAT TCGCTTAATG GTTGAAACAA ATGGAATATT GGGGTACTAT 2390

GCACGCATCT CGCTGGGTGA GCTTTCGT 2418

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 520 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Polyporus pinsitus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ser Arg Phe His Ser Leu Leu Ala Phe Val Val Ala Ser Leu Thr
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Ala Val Ala His Ala Gly Ile Gly Pro Val Ala Asp Leu Thr Ile Thr
20 25 30

Asn Ala Ala Val Ser Pro Asp Gly Phe Ser Arg Gln Ala Val Val Val
35 40 45

Asn Gly Gly Thr Pro Gly Pro Leu Ile Thr Gly Asn Met Gly Asp Arg
50 55 60

Phe Gln Leu Asn Val Ile Asp Asn Leu Thr Asn His Thr Met Val Lys
65 70 75 80

Ser Thr Ser Ile His Trp His Gly Phe Phe Gln Lys Gly Thr Asn Trp
85 90 95

Ala Asp Gly Pro Ala Phe Ile Asn Gln Cys Pro Ile Ser Ser Gly His
100 105 110

Ser Phe Leu Tyr Asp Phe Gln Val Pro Asp Gln Ala Gly Thr Phe Trp
115 120 125

Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro
130 135 140

Phe Val Val Tyr Asp Pro Asn Asp Pro Ala Ala Asp Leu Tyr Asp Val
145 150 155 160

Asp Asn Asp Asp Thr Val Ile Thr Leu Val Asp Trp Tyr His Val Ala
165 170 175

Ala Lys Leu Gly Pro Ala Phe Pro Leu Gly Ala Asp Ala Thr Leu Ile
180 185 190

Asn Gly Lys Gly Arg Ser Pro Ser Thr Thr Thr Ala Asp Leu Ser Val
195 200 205

Ile Ser Val Thr Pro Gly Lys Arg Tyr Arg Phe Arg Leu Val Ser Leu

210 215 220

Ser Cys Asp Pro Asn Tyr Thr Phe Ser Ile Asp Gly His Asn Met Thr
225 230 235 240

Ile Ile Glu Thr Asp Ser Ile Asn Thr Ala Pro Leu Val Val Asp Ser
245 250 255

Ile Gln Ile Phe Ala Ala Gln Arg Tyr Ser Phe Val Leu Glu Ala Asn
260 265 270

Gln Ala Val Asp Asn Tyr Trp Ile Arg Ala Asn Pro Asn Phe Gly Asn
275 280 285

Val Gly Phe Thr Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr Asp Gly
290 295 300

Ala Ala Ala Val Glu Pro Thr Thr Thr Gln Thr Thr Ser Thr Ala Pro
305 310 315 320

Leu Asn Glu Val Asn Leu His Pro Leu Val Thr Thr Ala Val Pro Gly
325 330 335

Ser Pro Val Ala Gly Gly Val Asp Leu Ala Ile Asn Met Ala Phe Asn
340 345 350

Phe Asn Gly Thr Asn Phe Phe Ile Asn Gly Thr Ser Phe Thr Pro Pro
355 360 365

Thr Val Pro Val Leu Leu Gln Ile Ile Ser Gly Ala Gln Asn Ala Gln
370 375 380

Asp Leu Leu Pro Ser Gly Ser Val Tyr Ser Leu Pro Ser Asn Ala Asp
385 390 395 400

Ile Glu Ile Ser Phe Pro Ala Thr Ala Ala Ala Pro Gly Ala Pro His
405 410 415

Pro Phe His Leu His Gly His Ala Phe Ala Val Val Arg Ser Ala Gly
420 425 430

Ser Thr Val Tyr Asn Tyr Asp Asn Pro Ile Phe Arg Asp Val Val Ser
435 440 445

Thr Gly Thr Pro Ala Ala Gly Asp Asn Val Thr Ile Arg Phe Arg Thr
450 455 460

Asp Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu
465 470 475 480

Glu Ala Gly Phe Ala Val Val Phe Ala Glu Asp Ile Pro Asp Val Ala
485 490 495

Ser Ala Asn Pro Val Pro Gln Ala Trp Ser Asp Leu Cys Pro Thr Tyr
500 505 510

Asp Ala Leu Asp Pro Ser Asp Gln
515 520

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2880 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: intron

(B) LOCATION: 544..592

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 837..899

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1014..1066

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1133..1187

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1284..1342

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1752..1815

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1873..1928

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2136..2195

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(364..543, 593..661, 716..835, 900..1013, 1067..1132, 1188..1283, 1343..1498, 1554..1751, 1816..1872, 1929..2135, 2196..2489)

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 662..715

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1499..1553

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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ACTGGAAGAG AACACCGAGG TCATGCATTC TGGCCAAGTG CGGCCAAAGG ACCGCTCGCT      240

GGTGCGGATA CTTAAAGGGC GGCGCGGGGA GGCCTGTCTA CCAAGCTCAA GCTCGCCTTG      300

GGTTCCCAGT CTCCGCCACC CTCCTCTTCC CCCACACAGT CGCTCCATAG CACCGTCGGC      360

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    Met Gly Leu Gln Arg Phe Ser Phe Phe Val Thr Leu Ala Leu Val
      1               5                  10                  15

GCT CGC TCT CTT GCA GCC ATC GGG CCG GTG GCG AGC CTC GTC GTC GCG        456
Ala Arg Ser Leu Ala Ala Ile Gly Pro Val Ala Ser Leu Val Val Ala
                20                  25                  30

AAC GCC CCC GTC TCG CCC GAC GGC TTC CTT CGG GAT GCC ATC GTG GTC        504
Asn Ala Pro Val Ser Pro Asp Gly Phe Leu Arg Asp Ala Ile Val Val
            35                  40                  45
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AAC GGC GTG GTC CCT TCC CCG CTC ATC ACC GGG AAG AAG GTCGGCGTGT 553
Asn Gly Val Val Pro Ser Pro Leu Ile Thr Gly Lys Lys
50 55 60

TCGTCGTCGT CCTACTCCTT TGCTGACAGC GATCTACAG GGA GAC CGC TTC CAG 607
Gly Asp Arg Phe Gln
65

CTC AAC GTC GTC GAC ACC TTG ACC AAC CAC AGC ATG CTC AAG TCC ACT 655
Leu Asn Val Val Asp Thr Leu Thr Asn His Ser Met Leu Lys Ser Thr
70 75 80

AGT ATC GTAAGTGTGA CGATCCGAAT GTGACATCAA TCGGGGCTAA TTAACCGCGC 711
Ser Ile

ACAG CAC TGG CAC GGC TTC TTC CAG GCA GGC ACC AAC TGG GCA GAA GGA 760
His Trp His Gly Phe Phe Gln Ala Gly Thr Asn Trp Ala Glu Gly
85 90 95

CCC GCG TTC GTC AAC CAG TGC CCT ATT GCT TCC GGG CAT TCA TTC CTG 808
Pro Ala Phe Val Asn Gln Cys Pro Ile Ala Ser Gly His Ser Phe Leu
100 105 110

TAC GAC TTC CAT GTG CCC GAC CAG GCA GTAAGCAGGA TTTTCTGGGG 855
Tyr Asp Phe His Val Pro Asp Gln Ala
115 120

TCCCCGTGTG ATGCAATGTT CTCATGCTCC GACGTGATCG ACAG GGG ACG TTC TGG 911
Gly Thr Phe Trp
125

TAC CAC AGT CAT CTG TCT ACG CAG TAC TGT GAC GGG CTG CGG GGG CCG 959
Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro
130 135 140

TTC GTC GTG TAC GAC CCC AAG GAC CCG CAC GCC AGC CGT TAC GAT GTT 1007
Phe Val Val Tyr Asp Pro Lys Asp Pro His Ala Ser Arg Tyr Asp Val
145 150 155

GAC AAT GTACGTGCGC CACGGAGTAT ATCACACAGC ATGCGTTGAC GTCGGGCCAA 1063
Asp Asn
160

CAG GAG AGC ACG GTC ATC ACG TTG ACC GAC TGG TAC CAC ACC GCT GCC 1111
Glu Ser Thr Val Ile Thr Leu Thr Asp Trp Tyr His Thr Ala Ala
165 170 175

CGG CTC GGT CCC AAG TTC CCA GTAAGCTCGC AATGGCTTAG TGTTCACAGG 1162
Arg Leu Gly Pro Lys Phe Pro
180

TTCTTTGCTT ATGTTGCTTC GATAG CTC GGC GCG GAC GCC ACG CTC ATC AAC 1214
Leu Gly Ala Asp Ala Thr Leu Ile Asn
185 190

GGT CTG GGG CGG TCG GCC TCG ACT CCC ACC GCT GCG CTT GCC GTG ATC 1262
Gly Leu Gly Arg Ser Ala Ser Thr Pro Thr Ala Ala Leu Ala Val Ile
195 200 205

AAC GTC CAG CAC GGA AAG CGC GTGAGCATTC TCTTGTATGC CATTTCAATG 1313
Asn Val Gln His Gly Lys Arg
210 215

CTTTGTGCTG ACCTATCGGA ACCGCGCAG TAC CGC TTC CGT CTC GTT TCG ATC 1366
Tyr Arg Phe Arg Leu Val Ser Ile
220

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TCG TGT GAC CCG AAC TAC ACG TTC AGC ATC GAC GGG CAC AAC CTG ACC      1414
Ser Cys Asp Pro Asn Tyr Thr Phe Ser Ile Asp Gly His Asn Leu Thr
     225                 230                 235

GTC ATC GAG GTC GAC GGC ATC AAT AGC CAG CCT CTC CTT GTC GAC TCT      1462
Val Ile Glu Val Asp Gly Ile Asn Ser Gln Pro Leu Leu Val Asp Ser
240                 245                 250             255

ATC CAG ATC TTC GCC GCA CAG CGC TAC TCC TTC GTG GTAAGTCCTG           1508
Ile Gln Ile Phe Ala Ala Gln Arg Tyr Ser Phe Val
                 260                 265

GCTTGTCGAT GCTCCAAAGT GGCCTCACTC ATATACTTTC GTTAG TTG AAT GCG        1562
                                                  Leu Asn Ala
                                                           270

AAT CAA ACG GTG GGC AAC TAC TGG GTT CGT GCG AAC CCG AAC TTC GGA      1610
Asn Gln Thr Val Gly Asn Tyr Trp Val Arg Ala Asn Pro Asn Phe Gly
                 275                 280                 285

ACG GTT GGG TTC GCC GGG GGG ATC AAC TCC GCC ATC TTG CGC TAC CAG      1658
Thr Val Gly Phe Ala Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr Gln
             290                 295                 300

GGC GCA CCG GTC GCC GAG CCT ACC ACG ACC CAG ACG CCG TCG GTG ATC      1706
Gly Ala Pro Val Ala Glu Pro Thr Thr Thr Gln Thr Pro Ser Val Ile
         305                 310                 315

CCG CTC ATC GAG ACG AAC TTG CAC CCG CTC GCG CGC ATG CCA GTG          1751
Pro Leu Ile Glu Thr Asn Leu His Pro Leu Ala Arg Met Pro Val
     320                 325                 330

GTATGTCTCT TTTTCTGATC ATCTGAGTTG CCCGTTGTTG ACCGCATTAT GTGTTACTAT    1811

CTAG CCT GGC AGC CCG ACA CCC GGG GGC GTC GAC AAG GCG CTC AAC CTC     1860
     Pro Gly Ser Pro Thr Pro Gly Gly Val Asp Lys Ala Leu Asn Leu
          335                   340             345

GCG TTT AAC TTC GTAAGTATCT CTACTACTTA GGCTGGAGGC TCGTCGCTGA          1912
Ala Phe Asn Phe
     350

TCATACGGTG CTTCAG AAC GGC ACC AAC TTC TTC ATC AAC AAC GCG ACT        1961
                  Asn Gly Thr Asn Phe Phe Ile Asn Asn Ala Thr
                          355                 360

TTC ACG CCG CCG ACC GTC CCG GTA CTC CTC CAG ATT CTG AGC GGT GCG      2009
Phe Thr Pro Pro Thr Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala
     365                 370                 375

CAG ACC GCA CAA GAC CTG CTC CCC GCA GGC TCT GTC TAC CCG CTC CCG      2057
Gln Thr Ala Gln Asp Leu Leu Pro Ala Gly Ser Val Tyr Pro Leu Pro
380                 385                 390                 395

GCC CAC TCC ACC ATC GAG ATC ACG CTG CCC GCG ACC GCC TTG GCC CCG      2105
Ala His Ser Thr Ile Glu Ile Thr Leu Pro Ala Thr Ala Leu Ala Pro
                 400                 405                 410

GGT GCA CCG CAC CCC TTC CAC CTG CAC GGT GTATGTTCCC CTGCCTTCCC        2155
Gly Ala Pro His Pro Phe His Leu His Gly
             415                 420

TTCTTATCCC CGAACCAGTG CTCACGTCCG TCCCATCTAG CAC GCC TTC GCG GTC      2210
                                            His Ala Phe Ala Val
                                                             425

GTT CGC AGC GCG GGG AGC ACC ACG TAT AAC TAC AAC GAC CCG ATC TTC      2258
Val Arg Ser Ala Gly Ser Thr Thr Tyr Asn Tyr Asn Asp Pro Ile Phe
             430                 435                 440
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CGC GAC GTC GTG AGC ACG GGC ACG CCC GCC GCG GGC GAC AAC GTC ACG 2306
Arg Asp Val Val Ser Thr Gly Thr Pro Ala Ala Gly Asp Asn Val Thr
445 450 455

ATC CGC TTC CAG ACG GAC AAC CCC GGG CCG TGG TTC CTC CAC TGC CAC 2354
Ile Arg Phe Gln Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys His
460 465 470

ATC GAC TTC CAC CTC GAC GCA GGC TTC GCG ATC GTG TTC GCA GAG GAC 2402
Ile Asp Phe His Leu Asp Ala Gly Phe Ala Ile Val Phe Ala Glu Asp
475 480 485 490

GTT GCG GAC GTG AAG GCG GCG AAC CCG GTT CCG AAG GCG TGG TCG GAC 2450
Val Ala Asp Val Lys Ala Ala Asn Pro Val Pro Lys Ala Trp Ser Asp
495 500 505

CTG TGC CCG ATC TAC GAC GGG CTG AGC GAG GCT AAC CAG TGAGCGGAGG 2499
Leu Cys Pro Ile Tyr Asp Gly Leu Ser Glu Ala Asn Gln
510 515

GCGTGGTGTT GAGCGTAAAG CTCGGGCGTC GACCTGGGGG GTTGAAGGTG TTCTGATTGA 2559

AATGGTCTTT GGGTTTATTT GTTGTTATTC TAACTCGGTT CTCTACGCAA GGACCGAGGA 2619

TTGTATAGGA TGAAGTAACT TCCCTAATGT ATTATGATAT CAATTGACGG AGGCATGGAC 2679

TGCGAAGTGT GTACAATGTG GTAGTGGTCT AGGCCTTGGA GACAAGCTGT GGATTTTTCT 2739

TGGGGGATGA AGAGGCGTGA AGGCTGAGAG CTATGCTATG CCTAGTGACG TGGTTATAGT 2799

AAATGTCCAT TACATTGACC AAGAACGACA AGAACCATAA GCTTGCTGAG GATAGATGGG 2859

GGCGCGTCCG CGAACGACTT G 2880

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 519 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Leu Gln Arg Phe Ser Phe Phe Val Thr Leu Ala Leu Val Ala
1 5 10 15

Arg Ser Leu Ala Ala Ile Gly Pro Val Ala Ser Leu Val Val Ala Asn
20 25 30

Ala Pro Val Ser Pro Asp Gly Phe Leu Arg Asp Ala Ile Val Val Asn
35 40 45

Gly Val Val Pro Ser Pro Leu Ile Thr Gly Lys Lys Gly Asp Arg Phe
50 55 60

Gln Leu Asn Val Val Asp Thr Leu Thr Asn His Ser Met Leu Lys Ser
65 70 75 80

Thr Ser Ile His Trp His Gly Phe Phe Gln Ala Gly Thr Asn Trp Ala
85 90 95

Glu Gly Pro Ala Phe Val Asn Gln Cys Pro Ile Ala Ser Gly His Ser
100 105 110

Phe Leu Tyr Asp Phe His Val Pro Asp Gln Ala Gly Thr Phe Trp Tyr
115 120 125

His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro Phe
130 135 140

Val Val Tyr Asp Pro Lys Asp Pro His Ala Ser Arg Tyr Asp Val Asp
145 150 155 160

Asn Glu Ser Thr Val Ile Thr Leu Thr Asp Trp Tyr His Thr Ala Ala
165 170 175

Arg Leu Gly Pro Lys Phe Pro Leu Gly Ala Asp Ala Thr Leu Ile Asn
180 185 190

Gly Leu Gly Arg Ser Ala Ser Thr Pro Thr Ala Ala Leu Ala Val Ile
195 200 205

Asn Val Gln His Gly Lys Arg Tyr Arg Phe Arg Leu Val Ser Ile Ser
210 215 220

Cys Asp Pro Asn Tyr Thr Phe Ser Ile Asp Gly His Asn Leu Thr Val
225 230 235 240

Ile Glu Val Asp Gly Ile Asn Ser Gln Pro Leu Leu Val Asp Ser Ile
245 250 255

Gln Ile Phe Ala Ala Gln Arg Tyr Ser Phe Val Leu Asn Ala Asn Gln
260 265 270

Thr Val Gly Asn Tyr Trp Val Arg Ala Asn Pro Asn Phe Gly Thr Val
275 280 285

Gly Phe Ala Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr Gln Gly Ala
290 295 300

Pro Val Ala Glu Pro Thr Thr Thr Gln Thr Pro Ser Val Ile Pro Leu
305 310 315 320

Ile Glu Thr Asn Leu His Pro Leu Ala Arg Met Pro Val Pro Gly Ser
325 330 335

Pro Thr Pro Gly Gly Val Asp Lys Ala Leu Asn Leu Ala Phe Asn Phe
340 345 350

Asn Gly Thr Asn Phe Phe Ile Asn Asn Ala Thr Phe Thr Pro Pro Thr
355 360 365

Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala Gln Thr Ala Gln Asp
370 375 380

Leu Leu Pro Ala Gly Ser Val Tyr Pro Leu Pro Ala His Ser Thr Ile
385 390 395 400

Glu Ile Thr Leu Pro Ala Thr Ala Leu Ala Pro Gly Ala Pro His Pro
405 410 415

Phe His Leu His Gly His Ala Phe Ala Val Val Arg Ser Ala Gly Ser
420 425 430

Thr Thr Tyr Asn Tyr Asn Asp Pro Ile Phe Arg Asp Val Val Ser Thr
435 440 445

Gly Thr Pro Ala Ala Gly Asp Asn Val Thr Ile Arg Phe Gln Thr Asp
450 455 460

Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Asp
465 470 475 480

Ala Gly Phe Ala Ile Val Phe Ala Glu Asp Val Ala Asp Val Lys Ala
485 490 495

Ala Asn Pro Val Pro Lys Ala Trp Ser Asp Leu Cys Pro Ile Tyr Asp
500 505 510

Gly Leu Ser Glu Ala Asn Gln
515

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3102 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Polyporus pinsitus

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 666..720

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 790..845

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1125..1182

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1390..1450

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1607..1661

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1863..1918

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1976..2025

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2227..2285

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2403..2458

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2576..2627

(ix) FEATURE:
(A) NAME/KEY: CDS

(B) LOCATION: join (665..721, 789..846, 1124..1183, 1389..1451, 1606..1662, 1862..1919, 1975..2026, 2226..2286, 2402..2459, 2575..2628).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTTCCCGACT AAACCAATCT CAGNCCGCTT CCTCCTAGGG AACCGAGCGA TGTGGCGGCC 60

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CTCTCTATCC AAGCTGTCCA TAAGAAGACG TTCAAATGCC GCAGCAAGCG AGGAAATAAG         120

CATCTAACAG TGTTTTTCCC ATAGTCGCAT TTGCGCCGCC TGTCGGACCG ACGCCCCTAG         180

AGCGCTTTGG GAAACGTCGC AAGTGGCGGG TGTTATTCGT GTAGACGAGA CGGTATTTGT         240

CTCATCATTC CCGTGCTTCA GGTTGACACA GCCCAAAGGT CTATGTACGG CCCTTCACAT         300

TCCCTGACAC ATTGACGCAA CCCTCGGTGC GCCTCCGACA GTGCCTCGGT TGTAGTATCG         360

GGACGCCCTA GGATGCAAGA TTGGAAGTCA CCAAGGCCCG AAGGGTATAA AATACCGAGA         420

GGTCCTACCA CTTCTGCATC TCCAGTCGCA GAGTTCCTCT CCCTTGCCAG CCACAGCTCG         480

AG ATG TCC TTC TCT AGC CTT CGC CGT GCC TTG GTC TTC CTG GGT GCT            527
   Met Ser Phe Ser Ser Leu Arg Arg Ala Leu Val Phe Leu Gly Ala
   1               5                  10                  15

TGC AGC AGT GCG CTG GCC TCC ATC GGC CCA GTC ACT GAG CTC GAC ATC           575
Cys Ser Ser Ala Leu Ala Ser Ile Gly Pro Val Thr Glu Leu Asp Ile
            20                  25                  30

GTT AAC AAG GTC ATC GCC CCG GAT GGC GTC GCT CGT GAT ACA GTC CTC           623
Val Asn Lys Val Ile Ala Pro Asp Gly Val Ala Arg Asp Thr Val Leu
        35                  40                  45

GCC GGG GGC ACG TTC CCG GGC CCA CTC ATC ACA GGA AAG AAG                   665
Ala Gly Gly Thr Phe Pro Gly Pro Leu Ile Thr Gly Lys Lys
    50                  55                  60

GTATGCTAAG TAGTCCCGCC CCCATCATCC TGTGGCTGAC GTTCGACGCC GCCAG              720

GGT GAC AAC TTC CGC ATC AAC GTC GTC GAC AAG TTG GTT AAC CAG ACT           768
Gly Asp Asn Phe Arg Ile Asn Val Val Asp Lys Leu Val Asn Gln Thr
        65                  70                  75

ATG CTG ACA TCC ACC ACC ATT GTATGTCACT AGCTCTCGCT ATCTCGAGAC              819
Met Leu Thr Ser Thr Thr Ile
    80

CCGCTGACCG ACAACATTTG CCGTAG CAC TGG CAC GGG ATG TTC CAG CAT              859
                             His Trp His Gly Met Phe Gln His
                             85                  90

ACG ACG AAC TGG GCG GAT GGT CCC GCC TTT GTG ACT CAA TGC CCT ATC           917
Thr Thr Asn Trp Ala Asp Gly Pro Ala Phe Val Thr Gln Cys Pro Ile
    95                  100                 105

ACC ACT GGT GAT GAT TTC CTG TAC AAC TTC CGC GTG CCC GAC CAG ACA           965
Thr Thr Gly Asp Asp Phe Leu Tyr Asn Phe Arg Val Pro Asp Gln Thr
    110                 115                 120

GTACGCAAAG GGCAGCATGC GTACTCAAAG ACATCTCTAA GCATTTGCTA CCTAG             1020

GGA ACG TAC TGG TAC CAT AGC CAT CTG GCC TTG CAG TAC TGT GAT GGG          1068
Gly Thr Tyr Trp Tyr His Ser His Leu Ala Leu Gln Tyr Cys Asp Gly
125                 130                 135                 140

CTT CGC GGC CCC CTG GTG ATT TAC GAT CCC CAT GAT CCG CAG GCA TAC          1116
Leu Arg Gly Pro Leu Val Ile Tyr Asp Pro His Asp Pro Gln Ala Tyr
            145                 150                 155

CTG TAT GAC GTC GAT GAC GTACGCAGCA CAGTTTCCCT AAAACGGTTA                 1164
Leu Tyr Asp Val Asp Asp
        160

ACTTCTAATT CTGTAAATAT CTTCATAG GAG AGC ACC GTT ATC ACT CTG               1213
                               Glu Ser Thr Val Ile Thr Leu
                                           165
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GCA GAC TGG TAC CAT ACC CCG GCG CCT CTG CTG CCG CCT GCC GCG 1258
Ala Asp Trp Tyr His Thr Pro Ala Pro Leu Leu Pro Pro Ala Ala
170 175 180

GTACGCCTCC ACACATCTGC ACAGCGTTCC GTATCTCATA CCCTTAAAGT TTATCGGACA 1318

ACT TTG ATT AAT GGC CTG GGT CGC TGG CCT GGC AAC CCC ACC GCC GAC 1366
Thr Leu Ile Asn Gly Leu Gly Arg Trp Pro Gly Asn Pro Thr Ala Asp
185 190 195 200

CTA GCC GTC ATC GAA GTC CAG CAC GGA AAG CGC GTATGTCATA GCTCGGTTAT 1419
Leu Ala Val Ile Glu Val Gln His Gly Lys Arg
205 210

CTATTCATAC TCGCGGCCTC GAAGCTAAAA CCTTGTTCCA G TAC CGG TTC CGA 1472
Tyr Arg Phe Arg
215

CTG GTC AGC ACC TCA TGC GAC CCC AAC TAC AAC TTC ACT ATC GAT GGC 1520
Leu Val Ser Thr Ser Cys Asp Pro Asn Tyr Asn Phe Thr Ile Asp Gly
220 225 230

CAC ACC ATG ACA ATC ATC GAG GCG GAT GGG CAG AAC ACC CAG CCA CAC 1568
His Thr Met Thr Ile Ile Glu Ala Asp Gly Gln Asn Thr Gln Pro His
235 240 245

CAA GTC GAC GGA CTT CAG ATC TTC GCG GCA CAG CGG TAC TCC TTC GTT 1616
Gln Val Asp Gly Leu Gln Ile Phe Ala Ala Gln Arg Tyr Ser Phe Val
250 255 260

GTATGTTTTC CGCATTTCGG GAAAAGGAAT TGCGCTGACA GCTCGAGTGT GCGTAG 1672

CTT AAC GCT AAC CAA GCG GTC AAC AAC TAC TGG ATC CGT GCG AAC CCT 1720
Leu Asn Ala Asn Gln Ala Val Asn Asn Tyr Trp Ile Arg Ala Asn Pro
265 270 275

AAC CGT GCT AAC ACT ACG GGC TTC GCC AAC GGC ATC AAC TCC GCC ATC 1768
Asn Arg Ala Asn Thr Thr Gly Phe Ala Asn Gly Ile Asn Ser Ala Ile
280 285 290 295

CTG CGC TAC AAG GGG GCG CCG ATT AAG GAG CCT ACG ACG AAC CAG ACT 1816
Leu Arg Tyr Lys Gly Ala Pro Ile Lys Glu Pro Thr Thr Asn Gln Thr
300 305 310

ACC ATC CGG AAC TTT TTG TGG GAG ACG GAC TTG CAC CCG CTC ACT GAC 1864
Thr Ile Arg Asn Phe Leu Trp Glu Thr Asp Leu His Pro Leu Thr Asp
315 320 325

CCA CGT GCA GTAAGTTCTA CACAGTCACC AACGGTGAGC TGTTGTCTGA 1913
Pro Arg Ala
330

TTGCACTGTG TTATAG CCT GGC CTT CCT TTC AAG GGG GGC GTT GAC CAC 1962
Pro Gly Leu Pro Phe Lys Gly Gly Val Asp His
335 340

GCT TTG AAC CTC AAC CTC ACT TTC GTACGTAGCG CCTCAGATAT CGAGTAGTCT 2016
Ala Leu Asn Leu Asn Leu Thr Phe
345

ATCTCCTGAC CGATTGACAG AAT GGA TCG GAG TTC TTC ATC AAC GAT GCG 2066
Asn Gly Ser Glu Phe Phe Ile Asn Asp Ala
350 355

CCT TTC GTC CCT CCG ACT GTC CCG GTG CTA CTG CAG ATC CTG AAC GGA 2114
Pro Phe Val Pro Pro Thr Val Pro Val Leu Leu Gln Ile Leu Asn Gly
360 365 370 375

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ACG CTC GAC GCG AAC GAC CTC CTG CCG CCC GGC AGC GTC TAC AAC CTT        2162
Thr Leu Asp Ala Asn Asp Leu Leu Pro Pro Gly Ser Val Tyr Asn Leu
                380                     385                     390

CCT CCG GAC TCC ACC ATC GAG CTG TCC ATT CCC GGA GGT GTG ACG GGT        2210
Pro Pro Asp Ser Thr Ile Glu Leu Ser Ile Pro Gly Gly Val Thr Gly
            395                     400                     405

GGC CCG CAC CCA TTC CAT TTG CAC GGG GTAATAATCT CTCTTTATAC              2257
Gly Pro His Pro Phe His Leu His Gly
        410                     415

TTTGGTCTCC CGATGCTGAC TTTCACTGCT CATCTTCAG CAC GCT TTC TCC GTC         2311
                                          His Ala Phe Ser Val
                                                      420

GTG CGT AGC GCC GGC AGC ACC GAA TAC AAC TAC GCG AAC CCG GTG AAG        2359
Val Arg Ser Ala Gly Ser Thr Glu Tyr Asn Tyr Ala Asn Pro Val Lys
            425                     430                     435

CGC GAC ACG GTC AGC ATT GGT CTT GCG GGC GAC AAC GTC ACC GTG CGC        2407
Arg Asp Thr Val Ser Ile Gly Leu Ala Gly Asp Asn Val Thr Val Arg
        440                     445                     450

TTC GTG GTATGTTTTA CAGCCTCTCT ATCTCCGTGG GCGTTCGGAA GTTGACTGGG         2463
Phe Val
    455

GCGTAG ACC GAC AAC CCC GGC CCG TGG TTC CTC CAC TGT CAC ATC GAC         2511
       Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp
                       460                     465

TTC CAT TTG CAA GCA GGC CTC GCC ATC GTG TTC GCG GAG GAC GCG CAG        2559
Phe His Leu Gln Ala Gly Leu Ala Ile Val Phe Ala Glu Asp Ala Gln
470                 475                     480                 485

GAC ACG AAG CTT GTG AAC CCC GTC CCT GTACGTCTTC TGGATGCATG              2606
Asp Thr Lys Leu Val Asn Pro Val Pro
                490

CGCTCCGCAC AGTGACTCAT CTTTTGCAAC AG GAG GAC TGG AAC AAG CTG TGC        2659
                                    Glu Asp Trp Asn Lys Leu Cys
                                    495                     500

CCC ACC TTC GAT AAG GCG ATG AAC ATC ACG GTT TGAGCGATGC                 2702
Pro Thr Phe Asp Lys Ala Met Asn Ile Thr Val
            505                     510

GTGGCGCTCA TGGTCATTTT CTTGGAATCT TTGCATAGGG CTGCAGCACG CTGGATACTC      2762

TTTCCCTTAG CAGGATATTA TTTAATGACC CCTGCGTTTA GTGCTTAGTT AGCTTTACTA      2822

CTGGTTGTAA TGTACGCAGC ATGCGTAATT CGGATAATGC TATCAATGTG TATATTATGA      2882

CACGCGTCAT GCGCGATGCT TGAGTTGCAA GGTCGGTTTC CGATGCTCGA CATAAACGTT      2942

TCACTTACAT ACACATTGGG TCTAGAACTG GATCTATCCA TGTATACAAA AACTCCTCAT      3002

ACAGCTGACT GGGGCGCTCT AGAGCATGGG TCCGATTGAT CAGATGTCGC GAACACGAGC      3062

CTCCTGAGCT CGAGGACTCT GAGAAGCGGC GGTGCGTTCT                            3102
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(2) INFORMATION FOR SEQ ID NO: 6

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 512 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Polyporus pinsitus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
Met Ser Phe Ser Ser Leu Arg Arg Ala Leu Val Phe Leu Gly Ala Cys
1               5                   10                  15

Ser Ser Ala Leu Ala Ser Ile Gly Pro Val Thr Glu Leu Asp Ile Val
            20                  25                  30

Asn Lys Val Ile Ala Pro Asp Gly Val Ala Arg Asp Thr Val Leu Ala
        35                  40                  45

Gly Gly Thr Phe Pro Gly Pro Leu Ile Thr Gly Lys Lys Gly Asp Asn
    50                  55                  60

Phe Arg Ile Asn Val Val Asp Lys Leu Val Asn Gln Thr Met Leu Thr
65                  70                  75                  80

Ser Thr Thr Ile His Trp His Gly Met Phe Gln His Thr Thr Asn Trp
                85                  90                  95

Ala Asp Gly Pro Ala Phe Val Thr Gln Cys Pro Ile Thr Thr Gly Asp
            100                 105                 110

Asp Phe Leu Tyr Asn Phe Arg Val Pro Asp Gln Thr Gly Thr Tyr Trp
        115                 120                 125

Tyr His Ser His Leu Ala Leu Gln Tyr Cys Asp Gly Leu Arg Gly Pro
    130                 135                 140

Leu Val Ile Tyr Asp Pro His Asp Pro Gln Ala Tyr Leu Tyr Asp Val
145                 150                 155                 160

Asp Asp Glu Ser Thr Val Ile Thr Leu Ala Asp Trp Tyr His Thr Pro
                165                 170                 175

Ala Pro Leu Leu Pro Pro Ala Ala Thr Leu Ile Asn Gly Leu Gly Arg
            180                 185                 190

Trp Pro Gly Asn Pro Thr Ala Asp Leu Ala Val Ile Glu Val Gln His
        195                 200                 205

Gly Lys Arg Tyr Arg Phe Arg Leu Val Ser Thr Ser Cys Asp Pro Asn
    210                 215                 220

Tyr Asn Phe Thr Ile Asp Gly His Thr Met Thr Ile Ile Glu Ala Asp
225                 230                 235                 240

Gly Gln Asn Thr Gln Pro His Gln Val Asp Gly Leu Gln Ile Phe Ala
                245                 250                 255

Ala Gln Arg Tyr Ser Phe Val Leu Asn Ala Asn Gln Ala Val Asn Asn
            260                 265                 270

Tyr Trp Ile Arg Ala Asn Pro Asn Arg Ala Asn Thr Thr Gly Phe Ala
        275                 280                 285

Asn Gly Ile Asn Ser Ala Ile Leu Arg Tyr Lys Gly Ala Pro Ile Lys
    290                 295                 300

Glu Pro Thr Thr Asn Gln Thr Thr Ile Arg Asn Phe Leu Trp Glu Thr
305                 310                 315                 320
```

Asp Leu His Pro Leu Thr Asp Pro Arg Ala Pro Gly Leu Pro Phe Lys
                325   330   335

Gly Gly Val Asp His Ala Leu Asn Leu Asn Leu Thr Phe Asn Gly Ser
            340   345   350

Glu Phe Phe Ile Asn Asp Ala Pro Phe Val Pro Pro Thr Val Pro Val
        355   360   365

Leu Leu Gln Ile Leu Asn Gly Thr Leu Asp Ala Asn Asp Leu Leu Pro
    370   375   380

Pro Gly Ser Val Tyr Asn Leu Pro Pro Asp Ser Thr Ile Glu Leu Ser
385   390   395   400

Ile Pro Gly Gly Val Thr Gly Gly Pro His Pro Phe His Leu His Gly
                405   410   415

His Ala Phe Ser Val Val Arg Ser Ala Gly Ser Thr Glu Tyr Asn Tyr
            420   425   430

Ala Asn Pro Val Lys Arg Asp Thr Val Ser Ile Gly Leu Ala Gly Asp
        435   440   445

Asn Val Thr Val Arg Phe Val Thr Asp Asn Pro Gly Pro Trp Phe Leu
    450   455   460

His Cys His Ile Asp Phe His Leu Gln Ala Gly Leu Ala Ile Val Phe
465   470   475   480

Ala Glu Asp Ala Gln Asp Thr Lys Leu Val Asn Pro Val Pro Glu Asp
                485   490   495

Trp Asn Lys Leu Cys Pro Thr Phe Asp Lys Ala Met Asn Ile Thr Val
            500   505   510

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2860 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 851..905

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1266..1320

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1351..1376

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1416..1468

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1625..1683

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1882..1934

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2202..2252

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2370..2425

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2543..2599

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(540..725, 782..850, 906..1025, 1086..1265, 1321..1350, 1377..1415, 1469..1624, 1684..1881, 1935..2201, 2253..2369, 2426..2542, 2600..2653)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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GGGGGGCGCG TCAATGGTCC GTTTGCGAAC ACATATGCAG GATAAACAGT GCGAAATATC      60

AATGTGGCGG CGACACAACC TCGCCGGCCG ACACTCGACG CTGTTGATCA TGATCATGTC     120

TTGTGAGCAT TCTATACGCA GCCTTGGAAA TCTCAGGCGA ATTTGTCTGA ATTGCGCTGG     180

GAGGCTGGCA GCGCAGATCG GTGTGTCGGT GCAGTAGCCG ACGCAGCACC TGGCGGAAGC     240

CGACATCTCG GGTACGACTT GATCTCCGCC AGATCACTGC GGTTCCGCCA TCGGCCGCGG     300

GGCCCATTCT GTGTGTGCGC TGTAGCACTC TGCATTCAGG CTCAACGTAT CCATGCTAGA     360

GGACCGTCCA GCTGTTGGCG CACGATTCGC GCAGAAAGCT GTACAGGCAG ATATAAGGAT     420

GTCCGTCCGT CAGAGACTCG TCACTCACAA GCCTCTTTTC CTCTTCGCCT TTCCAGCCTC     480

TTCCAACGCC TGCCATCGTC CTCTTAGTTC GCTCGTCCAT TCTTTCTGCG TAGTTAATC      539

ATG GGC AGG TTC TCA TCT CTC TGC GCG CTC ACC GCC GTC ATC CAC TCT       587
Met Gly Arg Phe Ser Ser Leu Cys Ala Leu Thr Ala Val Ile His Ser
  1               5                  10                  15

TTT GGT CGT GTC TCC GCC GCT ATC GGG CCT GTG ACC GAC CTC ACC ATC       635
Phe Gly Arg Val Ser Ala Ala Ile Gly Pro Val Thr Asp Leu Thr Ile
             20                  25                  30

TCC AAT GGG GAC GTT TCT CCC GAC GGC TTC ACT CGT GCC GCA GTG CTT       683
Ser Asn Gly Asp Val Ser Pro Asp Gly Phe Thr Arg Ala Ala Val Leu
         35                  40                  45

GCA AAC GGC GTC TTC CCG GGT CCT CTT ATC ACG GGA AAC AAG               725
Ala Asn Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys
     50                  55                  60

GTACGTGGCA TGCGTTCAGT CTACACCCTA CAAGCCTTCT AACTCTTTTA CCACAG         781

GGC GAC AAC TTC CAG ATC AAT GTT ATC GAC AAC CTC TCT AAC GAG ACG       829
Gly Asp Asn Phe Gln Ile Asn Val Ile Asp Asn Leu Ser Asn Glu Thr
         65                  70                  75

ATG TTG AAG TCG ACC TCC ATC GTATGTGCTT CTACTGCTTC TTAGTCTTGG          880
Met Leu Lys Ser Thr Ser Ile
     80                  85

CAATGGCTCA AGGTCTCCTC CGCAG CAT TGG CAC GGC TTC TTC CAG AAG GGT       932
                            His Trp His Gly Phe Phe Gln Lys Gly
                                            90

ACT AAC TGG GCT GAT GGA GCT GCC TTC GTC AAC CAG TGC CCT ATC GCG       980
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Thr Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro Ile Ala
 95                 100                 105                 110

ACG GGG AAC TCT TTC CTT TAC GAC TTC ACC GCG ACG GAC CAA GCA          1025
Thr Gly Asn Ser Phe Leu Tyr Asp Phe Thr Ala Thr Asp Gln Ala
                115                 120                 125

GTCAGTGCCT GTGGCGCTTA TGTTTTCCCG TAATCAGCAG CTAACACTCC GCACCCACAG    1085

GGC ACC TTC TGG TAC CAC AGT CAC TTG TCT ACG CAG TAC TGC GAT GGT      1133
Gly Thr Phe Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly
                130                 135                 140

TTG CGG GGC CCG ATG GTC GTA TAC GAC CCG AGT GAC CCG CAT GCG GAC      1181
Leu Arg Gly Pro Met Val Val Tyr Asp Pro Ser Asp Pro His Ala Asp
            145                 150                 155

CTT TAC GAC GTC GAC GAC GAG ACC ACG ATC ATC ACG CTC TCT GAT TGG      1229
Leu Tyr Asp Val Asp Asp Glu Thr Thr Ile Ile Thr Leu Ser Asp Trp
        160                 165                 170

TAT CAC ACC GCT GCT TCG CTC GGT GCT GCC TTC CCG GTAAGTTTAC           1275
Tyr His Thr Ala Ala Ser Leu Gly Ala Ala Phe Pro
    175                 180                 185

CCCAGCGCAC GGAGTTAAGA CCGGATCTAA CTGTAATACG TTCAG ATT GGC TCG        1329
                                                  Ile Gly Ser

GAC TCT ACC CTG ATT AAC GGC GTTGGCCGCT TCGCGGGTGG TGACAG ACT GAC     1382
Asp Ser Thr Leu Ile Asn Gly                              Thr Asp
    190                 195

CTT GCG GTT ATC ACT GTC GAG CAG GGC AAG CGC GTTAGTGATA CCCTCTACAG    1435
Leu Ala Val Ile Thr Val Glu Gln Gly Lys Arg
        200                 205

TTGACACTGT GCCATTGCTG ACAGTACTCT CAG TAC CGT ATG CGT CTT CTC TCG     1489
                                     Tyr Arg Met Arg Leu Leu Ser
                                         210                 215

CTG TCT TGC GAC CCC AAC TAT GTC TTC TCC ATT GAC GGC CAC AAC ATG      1537
Leu Ser Cys Asp Pro Asn Tyr Val Phe Ser Ile Asp Gly His Asn Met
                220                 225                 230

ACC ATC ATC GAG GCC GAC GCC GTC AAC CAC GAG CCC CTC ACG GTT GAC      1585
Thr Ile Ile Glu Ala Asp Ala Val Asn His Glu Pro Leu Thr Val Asp
            235                 240                 245

TCC ATC CAG ATC TAC GCC GGC CAA CGT TAC TCC TTC GTC GTACGTATTC       1634
Ser Ile Gln Ile Tyr Ala Gly Gln Arg Tyr Ser Phe Val
        250                 255                 260

CGAACAGCCA TGATCACGCC AAGCCCGATG CTAACGCGCC TACCCTCAG CTT ACC        1689
                                                      Leu Thr

GCT GAC CAG GAC ATC GAC AAC TAC TTC ATC CGT GCC CTG CCC AGC GCC      1737
Ala Asp Gln Asp Ile Asp Asn Tyr Phe Ile Arg Ala Leu Pro Ser Ala
        265                 270                 275

GGT ACC ACC TCG TTC GAC GGC GGC ATC AAC TCG GCT ATC CTG CGC TAC      1785
Gly Thr Thr Ser Phe Asp Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr
    280                 285                 290

TCT GGT GCC TCC GAG GTT GAC CCG ACG ACC ACG GAG ACC ACG AGC GTC      1833
Ser Gly Ala Ser Glu Val Asp Pro Thr Thr Thr Glu Thr Thr Ser Val
295                 300                 305                 310
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CTC CCC CTC GAC GAG GCG AAC CTC GTG CCC CTT GAC AGC CCC GCT GCT      1881
Leu Pro Leu Asp Glu Ala Asn Leu Val Pro Leu Asp Ser Pro Ala Ala
                315                 320                 325

GTACGTCGTA TTCTGCGCTT GCAAGGATCG CACATACTAA CATGCTCTTG TAG CCC       1937
                                                           Pro

GGT GAC CCC AAC ATT GGC GGT GTC GAC TAC GCG CTG AAC TTG GAC TTC      1985
Gly Asp Pro Asn Ile Gly Gly Val Asp Tyr Ala Leu Asn Leu Asp Phe
        330                 335                 340

AAC TTC GAT GGC ACC AAC TTC TTC ATC AAC GAC GTC TCC TTC GTG TCC      2033
Asn Phe Asp Gly Thr Asn Phe Phe Ile Asn Asp Val Ser Phe Val Ser
    345                 350                 355

CCC ACG GTC CCT GTC CTC CTC CAG ATT CTT AGC GGC ACC ACC TCC GCG      2081
Pro Thr Val Pro Val Leu Leu Gln Ile Leu Ser Gly Thr Thr Ser Ala
360                 365                 370                 375

GCC GAC CTT CTC CCC AGC GGT AGT CTC TTC GCG GTC CCG TCC AAC TCG      2129
Ala Asp Leu Leu Pro Ser Gly Ser Leu Phe Ala Val Pro Ser Asn Ser
                380                 385                 390

ACG ATC GAG ATC TCG TTC CCC ATC ACC GCG ACG AAC GCT CCC GGC GCG      2177
Thr Ile Glu Ile Ser Phe Pro Ile Thr Ala Thr Asn Ala Pro Gly Ala
            395                 400                 405

CCG CAT CCC TTC CAC TTG CAC GGT GTACGTGTCC CATCTCATAT GCTACGGAGC     2231
Pro His Pro Phe His Leu His Gly
        410                 415

TCCACGCTGA CCGCCCTATA G CAC ACC TTC TCT ATC GTT CGT ACC GCC GGC      2282
                        His Thr Phe Ser Ile Val Arg Thr Ala Gly
                                        420                 425

AGC ACG GAT ACG AAC TTC GTC AAC CCC GTC CGC CGC GAC GTC GTG AAC      2330
Ser Thr Asp Thr Asn Phe Val Asn Pro Val Arg Arg Asp Val Val Asn
                430                 435                 440

ACC GGT ACC GTC GGC GAC AAC GTC ACC ATC CGC TTC ACG GTACGCAGCA       2379
Thr Gly Thr Val Gly Asp Asn Val Thr Ile Arg Phe Thr
            445                 450

CTCTCCTAAC ATTCCCACTG CGCGATCACT GACTCCTCGC CCACAG ACT GAC AAC       2434
                                                   Thr Asp Asn
                                                   455

CCC GGC CCC TGG TTC CTC CAC TGC CAC ATC GAC TTC CAC TTG GAG GCC      2482
Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Glu Ala
        460                 465                 470

GGT TTC GCC ATC GTC TTC AGC GAG GAC ACC GCC GAC GTC TCG AAC ACG      2530
Gly Phe Ala Ile Val Phe Ser Glu Asp Thr Ala Asp Val Ser Asn Thr
    475                 480                 485

ACC ACG CCC TCG GTACGTTGTG CTCCCGTGCC CATCTCCGCG CGCCTGACTA          2582
Thr Thr Pro Ser
490

ACGAGCACCC CTTACAG ACT GCT TGG GAA GAT CTG TGC CCC ACG TAC AAC       2632
                   Thr Ala Trp Glu Asp Leu Cys Pro Thr Tyr Asn
                       495                 500

GCT CTT GAC TCA TCC GAC CTC TAATCGGTTC AAAGGGTCGC TCGCTACCTT         2683
Ala Leu Asp Ser Ser Asp Leu
505                 510
```

AGTAGGTAGA CTTATGCACC GGACATTATC TACAATGGAC TTTAATTTGG GTTAACGGCC 2743

GTTATACATA CGCGCACGTA GTATAAAGGT TCTCTGGATT GGTCGGACCT ACAGACTGCA 2803

ATTTTCGTGA CCTATCAACT GTATATTGAA GCACGACAGT GAATGGAAAT AGAGACA 2860

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 511 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Gly Arg Phe Ser Ser Leu Cys Ala Leu Thr Ala Val Ile His Ser
 1               5                  10                  15

Phe Gly Arg Val Ser Ala Ala Ile Gly Pro Val Thr Asp Leu Thr Ile
            20                  25                  30

Ser Asn Gly Asp Val Ser Pro Asp Gly Phe Thr Arg Ala Ala Val Leu
        35                  40                  45

Ala Asn Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys Gly Asp
    50                  55                  60

Asn Phe Gln Ile Asn Val Ile Asp Asn Leu Ser Asn Glu Thr Met Leu
65                  70                  75                  80

Lys Ser Thr Ser Ile His Trp His Gly Phe Phe Gln Lys Gly Thr Asn
                85                  90                  95

Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro Ile Ala Thr Gly
            100                 105                 110

Asn Ser Phe Leu Tyr Asp Phe Thr Ala Thr Asp Gln Ala Gly Thr Phe
        115                 120                 125

Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly
    130                 135                 140

Pro Met Val Val Tyr Asp Pro Ser Asp Pro His Ala Asp Leu Tyr Asp
145                 150                 155                 160

Val Asp Asp Glu Thr Thr Ile Ile Thr Leu Ser Asp Trp Tyr His Thr
                165                 170                 175

Ala Ala Ser Leu Gly Ala Ala Phe Pro Ile Gly Ser Asp Ser Thr Leu
            180                 185                 190

Ile Asn Gly Thr Asp Leu Ala Val Ile Thr Val Glu Gln Gly Lys Arg
        195                 200                 205

Tyr Arg Met Arg Leu Leu Ser Leu Ser Cys Asp Pro Asn Tyr Val Phe
    210                 215                 220

Ser Ile Asp Gly His Asn Met Thr Ile Ile Glu Ala Asp Ala Val Asn
225                 230                 235                 240

His Glu Pro Leu Thr Val Asp Ser Ile Gln Ile Tyr Ala Gly Gln Arg
                245                 250                 255

Tyr Ser Phe Val Leu Thr Ala Asp Gln Asp Ile Asp Asn Tyr Phe Ile
            260                 265                 270
```

Arg Ala Leu Pro Ser Ala Gly Thr Thr Ser Phe Asp Gly Gly Ile Asn
        275   280   285

Ser Ala Ile Leu Arg Tyr Ser Gly Ala Ser Glu Val Asp Pro Thr Thr
    290   295   300

Thr Glu Thr Thr Ser Val Leu Pro Leu Asp Glu Ala Asn Leu Val Pro
305   310   315   320

Leu Asp Ser Pro Ala Ala Pro Gly Asp Pro Asn Ile Gly Gly Val Asp
                325   330   335

Tyr Ala Leu Asn Leu Asp Phe Asn Phe Asp Gly Thr Asn Phe Phe Ile
            340   345   350

Asn Asp Val Ser Phe Val Ser Pro Thr Val Pro Val Leu Leu Gln Ile
        355   360   365

Leu Ser Gly Thr Thr Ser Ala Ala Asp Leu Leu Pro Ser Gly Ser Leu
    370   375   380

Phe Ala Val Pro Ser Asn Ser Thr Ile Glu Ile Ser Phe Pro Ile Thr
385   390   395   400

Ala Thr Asn Ala Pro Gly Ala Pro His Pro Phe His Leu His Gly His
                405   410   415

Thr Phe Ser Ile Val Arg Thr Ala Gly Ser Thr Asp Thr Asn Phe Val
            420   425   430

Asn Pro Val Arg Arg Asp Val Val Asn Thr Gly Thr Val Gly Asp Asn
        435   440   445

Val Thr Ile Arg Phe Thr Thr Asp Asn Pro Gly Pro Trp Phe Leu His
    450   455   460

Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile Val Phe Ser
465   470   475   480

Glu Asp Thr Ala Asp Val Ser Asn Thr Thr Thr Pro Ser Thr Ala Trp
                485   490   495

Glu Asp Leu Cys Pro Thr Tyr Asn Ala Leu Asp Ser Ser Asp Leu
            500   505   510

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2925 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Polyporus pinsitus

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 734..808

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 878..932

(ix) FEATURE:
(A) NAME/KEY: intron

(B) LOCATION: 1051..1104

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1219..1270

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1336..1397

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1713..7744

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2030..2085

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2308..2375

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2492..2569

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join (733..809, 877..933, 1050..1105, 1218..1271, 1335..1398, 1712..1775, 2029..2086, 2307..2376, 2492..2570). 2542..2600).

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```
CTCATAACTC TTCGCTTCTA GCATGGGGGC TGCGCACACC TGACAGACCC TTCGGGAGGC        60

GAACTCGAAT GCAGCGTACT CTATCNCACC TCCAGGAAAG GTAGGGATGG ACNCCGTGCA       120

CCAACAACTG TCTCTCCACC AGCAACCATC CCTTGGATAT GTCTCCACAC ACCCGGTGTC       180

TACAAGCGGG GATCTGTGCT GGTGAAGTGC TGTCTCCGGA GCGGCGGCGG CGAGCGACCA       240

GAACCCGAAC CAGTGCTAGT GCCCGACACC CGCGAGACAA TTGTGCAGGG TGAGTTATAT       300

TCTTCGTGAG ACGGCGCTGC GCGTCGGCAC TGAAAGCGTC GCAGTTAGGT GATGCAGCGG       360

TCCGCGCTAT TTTTGACGTC TGGCAGCTAT CCTAAGCCGC GCCTCCATAC ACCCCAGGCG       420

CTCTCGTTTG CTATAGGTAT AAATCCCTCA GCTTCAGAGC GTCGATCCTC ATCCCACACG       480

ACACCCGTTT CAGTCTTCTC GTAGCGCATT CCCTAGCCGC CCAGCCTCCG CTTTCGTTTT       540

CAAC ATG GGC AAG TAT CAC TCT TTT GTG AAC GTC GTC GCC CTT AGT CTT        589
     Met Gly Lys Tyr His Ser Phe Val Asn Val Val Ala Leu Ser Leu
     1               5                  10                  15

TCT TTG AGC GGT CGT GTG TTC GGC GCC ATT GGG CCC GTC ACC GAC TTG        637
Ser Leu Ser Gly Arg Val Phe Gly Ala Ile Gly Pro Val Thr Asp Leu
                20                  25                  30

ACT ATC TCT AAC GCC GAT GTT ACG CCT GAC GGC ATT ACT CGT GCT GCT        685
Thr Ile Ser Asn Ala Asp Val Thr Pro Asp Gly Ile Thr Arg Ala Ala
            35                  40                  45

GTC CTC GCG GGC GGC GTT TTC CCC GGG CCC CTC ATT ACC GGC AAC AAG        733
Val Leu Ala Gly Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys
        50                  55                  60

GTGAGCCGCG AAACCTTCTA CTAGCGCGCT CGTACGGTGC ACCGTTACTG AAGCCACACT       793
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TTGCGCTGTC AACAG GGG GAT GAA TTC CAG ATC AAT GTC ATC GAC AAC CTG 844
Gly Asp Glu Phe Gln Ile Asn Val Ile Asp Asn Leu
65 70 75

ACC AAC GAG ACC ATG TTG AAG TCG ACC ACA ATC GTAAGGTGCT TGCTCCCATA 897
Thr Asn Glu Thr Met Leu Lys Ser Thr Thr Ile
80 85

ATTAAGCCCG TCGCTGACTC GAAGTTTATC TGTAG CAC TGG CAT GGT ATC TTC 950
His Trp His Gly Ile Phe
90

CAG GCC GGC ACC AAC TGG GCA GAC GGC GCG GCC TTC GTG AAC CAG TGC 998
Gln Ala Gly Thr Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys
95 100 105

CCT ATC GCC ACG GGA AAC TCG TTC TTG TAC GAC TTC ACC GTT CCT GAT 1046
Pro Ile Ala Thr Gly Asn Ser Phe Leu Tyr Asp Phe Thr Val Pro Asp
110 115 120

CAA GCC GTACGTTTAT ACACTTCCCT TTCTGCGGCA TACTCTGACG CGCCGCTGGA 1102
Gln Ala
125

TCAG GGC ACC TTC TGG TAC CAC AGC CAC CTG TCC ACC CAG TAC TGT GAC 1151
Gly Thr Phe Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp
130 135 140

GGC CTG CGC GGT CCT CTT GTG GTC TAC GAC CCC GAC GAT CCC AAC GCG 1199
Gly Leu Arg Gly Pro Leu Val Val Tyr Asp Pro Asp Asp Pro Asn Ala
145 150 155

TCT CTT TAC GAC GTC GAT GAC GTAAGCAGGC TACTTGTGGA CTTGTATGGA 1250
Ser Leu Tyr Asp Val Asp Asp
160

TGTATCTCAC GCTCCCCTAC AG GAT ACT ACG GTT ATT ACG CTT GCG GAC TGG 1302
Asp Thr Thr Val Ile Thr Leu Ala Asp Trp
165 170

TAC CAC ACT GCG GCG AAG CTG GGC CCT GCC TTC CCC GTGAGTCTAC 1348
Tyr His Thr Ala Ala Lys Leu Gly Pro Ala Phe Pro
175 180 185

TCTTCCTCGT GTGTTAACAT AGGTGACGGC CGCTGATACG AGAGCTACCA G GCG GGT 1405
Ala Gly

CCG GAT AGC GTC TTG ATC AAT GGT CTT GGT CGG TTC TCC GGC GAT GGT 1453
Pro Asp Ser Val Leu Ile Asn Gly Leu Gly Arg Phe Ser Gly Asp Gly
190 195 200

GGA GGA GCG ACA AAC CTC ACC GTG ATC ACC GTC ACG CAA GGC AAA CGG 1501
Gly Gly Ala Thr Asn Leu Thr Val Ile Thr Val Thr Gln Gly Lys Arg
205 210 215 220

GTGAGTCCGC CCTGAGCTGG CCTCAATAGC GATATTGACG AGTCCATGCC CTCCCAG 1558

TAC CGC TTC CGC CTT GTG TCG ATC TCG TGC GAC CCC AAC TTC ACG TTC 1606
Tyr Arg Phe Arg Leu Val Ser Ile Ser Cys Asp Pro Asn Phe Thr Phe
225 230 235

TCG ATC GAC GGG CAC AAC ATG ACC ATC ATC GAG GTG GAC GGT GTC AAC 1654
Ser Ile Asp Gly His Asn Met Thr Ile Ile Glu Val Asp Gly Val Asn
240 245 250

CAC GAG GCC TTG GAC GTC GAC TCC ATT CAG ATT TTT GCG GGG CAG CGG 1702
His Glu Ala Leu Asp Val Asp Ser Ile Gln Ile Phe Ala Gly Gln Arg
255 260 265

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TAC TCC TTC ATC GTACGTTCCC TTGCCCTCGT GCTATATCCG CCCGTCTGCT        1754
Tyr Ser Phe Ile
    270

CACAGAGGCT TCTATATCGC AG CTC AAC GCC AAC CAG TCC ATC GAC AAC       1803
                         Leu Asn Ala Asn Gln Ser Ile Asp Asn
                                 275                 280

TAC TGG ATC CGC GCG ATC CCC AAC ACC GGT ACC ACC GAC ACC ACG GGC    1851
Tyr Trp Ile Arg Ala Ile Pro Asn Thr Gly Thr Thr Asp Thr Thr Gly
            285                 290                 295

GGC GTG AAC TCT GCT ATT CTT CGC TAC GAC ACC GCA GAA GAT ATC GAG    1899
Gly Val Asn Ser Ala Ile Leu Arg Tyr Asp Thr Ala Glu Asp Ile Glu
        300                 305                 310

CCT ACG ACC AAC GCG ACC ACC TCC GTC ATC CCT CTC ACC GAG ACG GAT    1947
Pro Thr Thr Asn Ala Thr Thr Ser Val Ile Pro Leu Thr Glu Thr Asp
    315                 320                 325

CTG GTG CCG CTC GAC AAC CCT GCG GCT CCC GGT GAC CCC CAG GTC GGC    1995
Leu Val Pro Leu Asp Asn Pro Ala Ala Pro Gly Asp Pro Gln Val Gly
330                 335                 340                 345

GGT GTT GAC CTG GCT ATG AGT CTC GAC TTC TCC TTC GTGAGTCCCA         2041
Gly Val Asp Leu Ala Met Ser Leu Asp Phe Ser Phe
            350                 355

CAGCACTCCG CGCCATTTCG CTTATTTACG CAGGAGTATT GTTCAG AAC GGT TCC     2096
                                                   Asn Gly Ser
                                                           360

AAC TTC TTT ATC AAC AAC GAG ACC TTC GTC CCG CCC ACA GTT CCC GTG    2144
Asn Phe Phe Ile Asn Asn Glu Thr Phe Val Pro Pro Thr Val Pro Val
            365                 370                 375

CTC CTG CAG ATT TTG AGT GGT GCG CAG GAC GCG GCG AGC CTG CTC CCC    2192
Leu Leu Gln Ile Leu Ser Gly Ala Gln Asp Ala Ala Ser Leu Leu Pro
            380                 385                 390

AAC GGG AGT GTC TAC ACA CTC CCT TCG AAC TCG ACC ATT GAG ATC TCG    2240
Asn Gly Ser Val Tyr Thr Leu Pro Ser Asn Ser Thr Ile Glu Ile Ser
        395                 400                 405

TTC CCC ATC ATC ACC ACC GAC GGT GTT CTG AAC GCG CCC GGT GCT CCG    2288
Phe Pro Ile Ile Thr Thr Asp Gly Val Leu Asn Ala Pro Gly Ala Pro
    410                 415                 420

CAC CCG TTC CAT CTC CAC GGC GTAAGTCCTT GCTTTCCTCA GTGCCTCGCT       2339
His Pro Phe His Leu His Gly
425                 430

TCCACGACGT CCACTGATCC CACACATCCC ATGTGCAG CAC ACC TTC TCG GTG      2392
                                          His Thr Phe Ser Val
                                                          435

GTG CGC AGC GCC GGG AGC TCG ACC TTC AAC TAC GCC AAC CCA GTC CGC    2440
Val Arg Ser Ala Gly Ser Ser Thr Phe Asn Tyr Ala Asn Pro Val Arg
            440                 445                 450

CGG GAC ACC GTC AGT ACT GGT AAC TCT GGC GAC AAC GTC ACT ATC CGC    2488
Arg Asp Thr Val Ser Thr Gly Asn Ser Gly Asp Asn Val Thr Ile Arg
        455                 460                 465

TTC ACG GTACGTCTTC TCCGGAGCCC TCCCACCCGT GTGTCCGCTG AGCGCTGAAC     2544
Phe Thr
    470

ACCGCCCACC GTGCTGCTGC TGCGCAG ACC GAC AAC CCA GGC CCG TGG TTC      2595
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Thr Asp Asn Pro Gly Pro Trp Phe
475

CTC CAC TGC CAC ATC GAC TTC CAC CTG GAG GCC GGC TTC GCC ATC GTC 2643
Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile Val
480 485 490

TGG GGG GAG GAC ACT GCG GAC ACC GCG TCC GCG AAT CCC GTT CCT 2688
Trp Gly Glu Asp Thr Ala Asp Thr Ala Ser Ala Asn Pro Val Pro
495 500 505

GTACGTCGTG CCTGCTGAGC TCTTTGTGCC CGAACAGGGT GCTGATCGTG CCTTCCTCCG 2748

TGCAG ACG GCG TGG AGC GAT TTG TGC CCC ACT TAC GAT GCT TTG GAC TCG 2798
Thr Ala Trp Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Ser
510 515 520

TCC GAC CTC TGATCGACAA GGCATGAAGG CTGAAGCAGC TGCGGTCAAT 2847
Ser Asp Leu
525

TCTCGAACAC ACTTTACTCG AACATTCATT TTTCTTTGGC TCGGGATCGG AACAAATCAT 2907

GGGGGGGCCG GACCGTCT 2925

(2) INFORMATION FOR SEQ ID NO: 10

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 527 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Polyporus pinsitus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met Gly Lys Tyr His Ser Phe Val Asn Val Val Ala Leu Ser Leu Ser
1 5 10 15

Leu Ser Gly Arg Val Phe Gly Ala Ile Gly Pro Val Thr Asp Leu Thr
20 25 30

Ile Ser Asn Ala Asp Val Thr Pro Asp Gly Ile Thr Arg Ala Ala Val
35 40 45

Leu Ala Gly Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys Gly
50 55 60

Asp Glu Phe Gln Ile Asn Val Ile Asp Asn Leu Thr Asn Glu Thr Met
65 70 75 80

Leu Lys Ser Thr Thr Ile His Trp His Gly Ile Phe Gln Ala Gly Thr
85 90 95

Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro Ile Ala Thr
100 105 110

Gly Asn Ser Phe Leu Tyr Asp Phe Thr Val Pro Asp Gln Ala Gly Thr
115 120 125

Phe Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg
130 135 140

Gly Pro Leu Val Val Tyr Asp Pro Asp Asp Pro Asn Ala Ser Leu Tyr
145 150 155 160

Asp Val Asp Asp Asp Thr Thr Val Ile Thr Leu Ala Asp Trp Tyr His
165 170 175

Thr Ala Ala Lys Leu Gly Pro Ala Phe Pro Ala Gly Pro Asp Ser Val
180 185 190

Leu Ile Asn Gly Leu Gly Arg Phe Ser Gly Asp Gly Gly Gly Ala Thr
195 200 205

Asn Leu Thr Val Ile Thr Val Thr Gln Gly Lys Arg Tyr Arg Phe Arg
210 215 220

Leu Val Ser Ile Ser Cys Asp Pro Asn Phe Thr Phe Ser Ile Asp Gly
225 230 235 240

His Asn Met Thr Ile Ile Glu Val Asp Gly Val Asn His Glu Ala Leu
245 250 255

Asp Val Asp Ser Ile Gln Ile Phe Ala Gly Gln Arg Tyr Ser Phe Ile
260 265 270

Leu Asn Ala Asn Gln Ser Ile Asp Asn Tyr Trp Ile Arg Ala Ile Pro
275 280 285

Asn Thr Gly Thr Thr Asp Thr Thr Gly Gly Val Asn Ser Ala Ile Leu
290 295 300

Arg Tyr Asp Thr Ala Glu Asp Ile Glu Pro Thr Thr Asn Ala Thr Thr
305 310 315 320

Ser Val Ile Pro Leu Thr Glu Thr Asp Leu Val Pro Leu Asp Asn Pro
325 330 335

Ala Ala Pro Gly Asp Pro Gln Val Gly Gly Val Asp Leu Ala Met Ser
340 345 350

Leu Asp Phe Ser Phe Asn Gly Ser Asn Phe Phe Ile Asn Asn Glu Thr
355 360 365

Phe Val Pro Pro Thr Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala
370 375 380

Gln Asp Ala Ala Ser Leu Leu Pro Asn Gly Ser Val Tyr Thr Leu Pro
385 390 395 400

Ser Asn Ser Thr Ile Glu Ile Ser Phe Pro Ile Ile Thr Thr Asp Gly
405 410 415

Val Leu Asn Ala Pro Gly Ala Pro His Pro Phe His Leu His Gly His
420 425 430

Thr Phe Ser Val Val Arg Ser Ala Gly Ser Ser Thr Phe Asn Tyr Ala
435 440 445

Asn Pro Val Arg Arg Asp Thr Val Ser Thr Gly Asn Ser Gly Asp Asn
450 455 460

Val Thr Ile Arg Phe Thr Thr Asp Asn Pro Gly Pro Trp Phe Leu His
465 470 475 480

Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile Val Trp Gly
485 490 495

Glu Asp Thr Ala Asp Thr Ala Ser Ala Asn Pro Val Pro Thr Ala Trp
500 505 510

Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Ser Ser Asp Leu
515 520 525

Applicant's or agent's file reference number 4185.204-WO	International application to be assigned PCT/US 95/07536

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13 *bis*)

A. The indications made below relate to the microorganism referred to in the description on page 55, line 4.

B. IDENTIFICATION OF — Further deposits are identified on an additional sheet ☒

Name of depository institution

Agricultural Research Service Patent Culture Collection (NRRL)

Address of depository institution (*including postal code and country*)

Northern Regional Research Center
1815 University Street
Peoria, IL 61604, US

Date of deposit	Accession Number
May 25, 1995	NRRL B-21263

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) — This information is continued on an additional sheet ☐

In respect of those designations in which a European and/or Australia Patent is sought, during the pendency of the patent application, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC/Regulation 3.25 of Australia Statutory Rule 1991 No. 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13 *bis*)

A. The indications made below relate to the microorganism referred to in the description on page 55, line 6.

B. IDENTIFICATION OF — Further deposits are identified on an additional sheet ☒

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1815 University Street
Peoria, IL 61604, US

Date of deposit	Accession Number
May 25, 1995	NRRL B-21268

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Applicant's or agent's file reference number 4185.204-WO	International application N to be assigned PCT/US 95/07536

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A. The indications made below relate to the microorganism referred to in the description on page 55, line 16.

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

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Date of deposit	Accession Number
May 25, 1995	NRRL B-21267

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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

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What we claim is:

1. A DNA construct containing a sequence encoding a *Polyporus* laccase.

2. The construct of Claim 1 which comprises a sequence encoding a *Polyporus pinsitus* laccase.

3. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.

4. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 1.

5. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 4.

6. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 3.

7. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 6.

8. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 5.

9. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 8.

10. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 7.

11. The construct of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 10.

12. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 9.

13. The construct of Claim 1, which comprises the nucleic acid sequence selected from those contained in NRRL B-21263, 21264, 21265, 21266, 21267, and 21268.

14. A substantially pure *Polyporus* laccase enzyme.

15. The enzyme of Claim 14 which is a *Polyporus pinsitus* laccase.

16. The enzyme of Claim 14 which comprises the amino acid sequence selected from the group consisting of the sequences depicted in SEQ ID NOS. 4, 6, 8, and 10 or a sequence with at least about 80% homology thereto.

17. A recombinant vector comprising an DNA construct containing a sequence encoding a *Polyporus* laccase.

18. The vector of Claim 17 in which the construct is operably linked to a promoter sequence.

19. The vector of Claim 18 in which the promoter is a fungal or yeast promoter.

20. The vector of Claim 19 in which the promoter is the TAKA amylase promoter of *Aspergillus oryzae*.

21. The vector of Claim 18 in which the promoter is the glucoamylase (glaA) promoter of *Aspergillus niger* or *Aspergillus awamori*.

22. The vector of Claim 17 which also comprises a selectable marker.

23. The vector of Claim 22 in which the selectable marker is selected from the group consisting of *amdS*, *pyrG*, *argB*, *nia*D, *sC*, *trpC* and *hygB*.

24. The vector of Claim 22 in which the selectable marker is the amdS marker of *Aspergillus nidulans* or *Aspergillus oryzae*, or the pyrG marker of *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus awamori*, or *Aspergillus oryzae*.

25. The vector of Claim 18 which comprises both the TAKA amylase promoter of *Aspergillus oryzae* and the amdS or pyrG marker of *Aspergillus nidulans* or *Aspergillus oryzae*.

26. A recombinant host cell comprising a heterologous DNA construct containing a sequence encoding a *Polyporus* laccase.

27. The cell of Claim 26 which is a fungal cell.

28. The cell of Claim 27 which is an *Aspergillus* cell.

29. The cell of Claim 26 in which the construct is integrated into the host cell genome.

30. The cell of Claim 26 in which the construct is contained on a vector.

31. The cell of Claim 26 which comprises a construct containing a sequence encoding an amino acid sequence selected from the group consisting of those depicted in SEQ ID NOS. 2, 4, 6, 8, and 10.

32. A method for obtaining a laccase enzyme which comprises culturing a recombinant host cell comprising a DNA construct containing a nucleic acid sequence encoding a *Polyporus* laccase enzyme, under conditions conducive to expression of the enzyme, and recovering the enzyme from the culture.

33. A method for obtaining a laccase enzyme which comprises culturing a recombinant *Aspergillus* host cell comprising a DNA construct containing a nucleic acid sequence encoding a *Polyporus*-like laccase enzyme, under conditions conducive to expression of the enzyme, and recovering the enzyme from the culture.

34. A *Polyporus* enzyme obtained by the method of Claim 33.

35. A method for polymerizing a lignin or lignosulfate substrate in solution which comprises contacting the substrate with a *Polyporus* laccase.

36. A method for in situ depolymerization in Kraft pulp which comprises contacting the pulp with a *Polyporus* laccase.

37. A method for oxidizing dyes or dye precursors which comprises contacting the dye or dye precursor with a *Polyporus* laccase.

38. A method for dyeing hair which comprises contacting a *Polyporus* laccase, in the presence or absence of at least one modifier, with at least one dye precursor, for a time and under conditions sufficient to permit oxidation of the dye precursor to a dye.

39. The method of claim 38 in which the dye precursor is selected from the group consisting of a diamine, aminophenol, and a phenol.

40. The method of claim 38, wherein the modifier, when used, is a meta-diamine, a meta-aminophenol or a polyphenol.

41. The method of claim 38 in which the dye precursor is a primary intermediate selected from the group consisting of an ortho- or para-diamine or aminophenol.

42. The method of claim 38 in which more than one dye precursor is used.

43. The method of claim 38 in which more than one modifier is used.

44. The method of claim 38 in which both a primary intermediate and a modifier are used.

45. A dye composition comprising a *Polyporus* laccase combined with at least one dye precursor.

46. A dye composition comprising a *Polyporus* laccase combined with at least one primary intermediate and at least one modifier.

47. A container containing a dye composition comprising a *Polyporus* laccase and at least one dye precursor in an oxygen-free atmosphere.

48. The container of claim 47 which contains at least one primary intermédiate dye precusor combined with at least one modifier.

49. A method of polymerizing or oxidizing a phenolic or aniline compound which comprises contacting the phenolic or aniline compound with a *Polyporus* laccase.

10 20 30 40 50 60 70

AGATTTCTGA CACCGGTGCA ATCTTGACAC TGTACCAACC GGGCAAGTCT CGTCCTTGGT TCTCGGGGAC

80 90 100 110 120 130 140

TGGCGCCGGT CGCTACCCCT TGGTCATTCA CTCTACCAGA GCGCTGGCTT CGCCGAGGTA TAAAGGATGT

150 160 170 180 190 200 210

TGCGCGACAC CCTCAACACC CCAACTCAAG CCCCACTTGA GCTTTTGCGA GATCCTCCAC ATACCACTCA

220 230 239 248 257 266

CTACTTTCAA GTTCTTCAAC ATG TCG AGG TTT CAC TCT CTT CTC GCT TTC GTC GTT
Met Ser Arg Phe His Ser Leu Leu Ala Phe Val Val

275 284 293 302 311 320

GCT TCC CTT ACG GCT GTG GCC CAC GCT GGT ATC GGT CCC GTC GCC GAC CTA ACC
Ala Ser Leu Thr Ala Val Ala His Ala Gly Ile Gly Pro Val Ala Asp Leu Thr

329 338 347 356 365 374

ATC ACC AAC GCA GCG GTC AGC CCC GAC GGG TTT TCT CGC CAG GCC GTC GTC GTG
Ile Thr Asn Ala Ala Val Ser Pro Asp Gly Phe Ser Arg Gln Ala Val Val Val

383 392 401 410 423 433

AAC GGC GGC ACC CCT GGC CCT CTC ATC ACG GGT AAC ATG GTTCGTCTCG GCTCGCACTA
Asn Gly Gly Thr Pro Gly Pro Leu Ile Thr Gly Asn MET

443 453 463 473 482 491

GGGGGTTGTA TCGTTCCTGA CGTTGTTGGA G GGG GAT CGC TTC CAG CTC AAT GTC ATC
Gly Asp Arg Phe Gln Leu Asn Val Ile

500 509 518 527 543 553

GAC AAC CTT ACC AAC CAC ACG ATG GTG AAG AGC ACG AGT ATT GTGAGCTGCT ATTTCTCCGG
Asp Asn Leu Thr Asn His Thr MET Val Lys Ser Thr Ser Ile

FIG.1A

563 573 583 592 601 610

ACGGGGCTTC ATTGTGCTAA TAATCGTCGT GTGCAG CAC TGG CAC GGT TTC TTC CAG AAG
His Trp His Gly Phe Phe Gln Lys

619 628 637 646 655 664

GGT ACC AAC TGG GCC GAC GGT CCC GCC TTC ATC AAC CAG TGC CCG ATC TCA TCT
Gly Thr Asn Trp Ala Asp Gly Pro Ala Phe Ile Asn Gln Cys Pro Ile Ser Ser

673 682 691 700 709 720

GGT CAC TCG TTC CTG TAC GAC TTC CAG GTT CCT GAC CAG GCT G GTAAGTACGG
Gly His Ser Phe Leu Tyr Asp Phe Gln Val Pro Asp Gln Ala Gly

730 740 750 760 770 779

TCGTTATGGA GTATACTGCG CATTGCTAAA CCACATGGTG AACAG GT ACC TTC TGG TAT
Thr Phe Trp Tyr

788 797 806 815 824 833

CAC AGT CAC TTG TCT ACG CAG TAC TGT GAT GGT TTG AGG GGT CCG TTC GTT GTT
His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro Phe Val Val

842 851 860 869 878 889

TAC GAC CCG AAT GAC CCG GCC GCC GAC CTG TAC GAC GTC GAC AAC G GTAAGGACGA
Tyr Asp Pro Asn Asp Pro Ala Ala Asp Leu Tyr Asp Val Asp Asn Asp

899 909 919 929 940 949

ATTCGAACCG TAAATACTTG CTTACTGATA CTTCTCGATG AATTAG AC GAC ACT GTC ATT
Asp Thr Val Ile

958 967 976 985 994 1009

ACC CTT GTG GAT TGG TAC CAC GTC GCC GCG AAG CTG GGC GGG GCA TTC CC GTAAGTCCAT
Thr Leu Val Asp Trp Tyr His Val Ala Ala Lys Leu Gly Pro Ala Phe Pro

FIG.1B

1019 1029 1039 1049 1060 1069

GAGTATTCTG CTGTTGAATC TGTCTTAACT GTGCATATCA G T CTC GGC GCC GAC GCC ACC
Leu Gly Ala Asp Ala Thr

1078 1087 1096 1105 1114 1123

CTC ATC AAC GGT AAG GGA CGC TCC CCC AGC ACG ACC ACC GCG GAC CTC TCA GTT
Leu Ile Asn Gly Lys Gly Arg Ser Pro Ser Thr Thr Thr Ala Asp Leu Ser Val

1132 1141 1156 1166 1176 1186

ATC AGC GTC ACC CCG GGT AAA CG GTATGCTATA TCTTATCTTA TCTGATGGCA TTTCTCTGAG
Ile Ser Val Thr Pro Gly Lys Arg

1196 1207 1216 1225 1234

ACATTCTCCA G C TAC CGT TTC CGC CTG GTG TCC CTG TCG TGC GAC CCC AAC TAC
Tyr Arg Phe Arg Leu Val Ser Leu Ser Cys Asp Pro Asn Tyr

1243 1252 1261 1270 1279 1288

ACG TTC AGC ATC GAT GGT CAC AAC ATG ACG ATC ATC GAG ACC GAC TCA ATC AAC
Thr Phe Ser Ile Asp Gly His Asn MET Thr Ile Ile Glu Thr Asp Ser Ile Asn

1297 1306 1315 1324 1333 1342

ACC GCG CCC CTC GTC GTC GAC TCC ATT CAG ATC TTC GCC CCC CAG CGT TAC TCC
Thr Ala Pro Leu Val Val Asp Ser Ile Gln Ile Phe Ala Ala Gln Arg Tyr Ser

1351 1364 1374 1384 1394 1404

TTC GTG GTAAGTTCGA TTCATCCTCT AACGTTGGTC GCTGTTAGTG ATCGTATGGT CATGTAG
Phe Val

1414 1423 1432 1441 1450 1459

CTC GAG GCC AAC CAG GCC GTC GAC AAC TAC TGG ATT CGC GCC AAC CCG AAC TTC
Leu Glu Ala Asn Gln Ala Val Asp Asn Tyr Trp Ile Arg Ala Asn Pro Asn Phe

FIG.1C

1468 1477 1486 1495 1504 1513

GGT AAC GTC GGG TTC ACC GGC GGC ATT AAC TCG GCT ATC CTC CGC TAC GAT GGT
Gly Asn Val Gly Phe Thr Gly Gly Ile Asn Ser Ala Ile Leu Arg Tyr Asp Gly

1522 1531 1540 1549 1558 1567

GCC GCT GCC GTG GAG CCC ACC ACA ACG CAA ACC ACG TCG ACT GCG CCG CTC AAC
Ala Ala Ala Val Glu Pro Thr Thr Thr Gln Thr Thr Ser Thr Ala Pro Leu Asn

1576 1585 1594 1603 1619 1629

GAG GTC AAC CTG CAC CCG CTG GTT ACC ACC GCT GTG GTATGTAATA TTGTCGGTAA
Glu Val Asn Leu His Pro Leu Val Thr Thr Ala Val

1639 1649 1659 1669 1678 1687

TGTAATACAT TGTTGCTGAC CTCGACCCCC ACAG CCT GGC TCG CCC GTC GCT GGT GGT
Pro Gly Ser Pro Val Ala Gly Gly

1696 1705 1714 1723 1732 1741

GTC GAC CTG GCC ATC AAC ATG GCG TTC AAC TTC AAC GGC ACC AAC TTC TTC ATC
Val Asp Leu Ala Ile Asn MET Ala Phe Asn Phe Asn Gly Thr Asn Phe Phe Ile

1750 1759 1768 1777 1786 1795

AAC GGC ACG TCT TTC ACG CCC CCG ACC GTG CCT GTC CTG CTC CAG ATC ATC AGC
Asn Gly Thr Ser Phe Thr Pro Pro Thr Val Pro Val Leu Leu Gln Ile Ile Ser

1804 1813 1822 1831 1840 1849

GGC GCG CAG AAC GCG CAG GAC CTC CTG CCC TCC GGT AGC GTC TAC TCG CTT CCC
Gly Ala Gln Asn Ala Gln Asp Leu Leu Pro Ser Gly Ser Val Tyr Ser Leu Pro

1858 1867 1876 1885 1894 1903

TCG AAC GCC GAC ATC GAG ATC TCC TTC CCC GCC ACC GCC GCC GCC CCC GGT GCG
Ser Asn Ala Asp Ile Glu Ile Ser Phe Pro Ala Thr Ala Ala Ala Pro Gly Ala

FIG.1D

1912 1921 1930 1939 1948 1957

CCC CAC CCC TTC CAC TTG CAC GGG CAC GCG TTC GCG GTC GTC CGC AGC GCC GGC
Pro His Pro Phe His Leu His Gly His Ala Phe Ala Val Val Arg Ser Ala Gly

1966 1975 1984 1993 2002 2011

AGC ACG GT TAC AAC TAC GAC AAC CCC ATC TTC CGC GAC TC GTC AGC ACG GGG
Ser Thr Val Tyr Asn Tyr Asp Asn Pro Ile Phe Arg Asp Val Val Ser Thr Gly

2020 2029 2038 2047 2056 2065

ACG CCT GCG GCC GGT GAC AAC GTC ACC ATC CGC TTC CGC ACC GAC AAC CCC GGC
Thr Pro Ala Ala Gly Asp Asn Val Thr Ile Arg Phe Arg Thr Asp Asn Pro Gly

2074 2083 2092 2101 2110 2119

CCG TGG TTC CTC CAC TGC CAC ATC GAC TTC CAC CTC GAG GCC GGC TTC GCC GTC
Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Val

2128 2137 2146 2155 2164 2173

GTG TTC GCG GAG GAC ATC CCC GAC GTC GCG TCG GCG AAC CCC GTC CCC CAG GCG
Val Phe Ala Glu Asp Ile Pro Asp Val Ala Ser Ala Asn Pro Val Pro Gln Ala

2182 2191 2200 2209 2218 2231

TGG TCC GAC CTC TGT CCG ACC TAC GAC GCG CTC GAC CCG AGC GAC CAG TAAATGGCTT
Trp Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Pro Ser Asp Gln

2241 2251 2261 2271 2281 2291 2301

GCGCCGGTCG ATGATAGGAT ATGGACGGTG AGTTCGCACT TGCAATACGG ACTCTCGCCT CATTATGGTT

2311 2321 2331 2341 2351 2361 2371

ACACACTCGC TCTGGATCTC TCGCCTGTCG ACAGAACAAA CTTGTATAAT TCGCTTAATG GTTGAAACAA

2381 2391 2401 2411

ATGGAATATT GGGGTACTAT GCACGCATCT CGCTGGGTGA GCTTTCGT

FIG.1E

10 20 30 40 50 60 70

GCGGCGCACA AACCGTGGGA GCCAACACAC TCCCGTCCAC TCTCACACTG GCCAGATTCG CGCGACCGCC

80 90 100 110 120 130 140

GCCTTTCAGG CCCAAACAGA TCTGGCAGGT TTCGATGGCG CACGCCGCCG TGCCTGCCGG ATTCAATTGT

150 160 170 180 190 200 210

GCGCCAGTCG GGCATCCGGA TGGCTCTACC AGCGCGGTTG ACTGGAAGAG AACACCGAGG TCATGCATTC

220 230 240 250 260 270 280

TGGCCAAGTG CGGCCAAAGG ACCGCTCGCT GGTGCGGATA CTTAAAGGGC GGCGCGGGA GGCCTGTCTA

290 300 310 320 330 340 350

CCAAGCTCAA GCTCGCCTTG GGTTCCCAGT CTCCGCCACC CTCCTCTTCC CCCACACAGT CGCTCCATAG

360 369 378 387 396 405

CACCGTCGGC GCC ATG GGT CTG CAG CGA TTC AGC TTC TTC GTC ACC CTC GCG CTC
MET Gly Leu Gln Arg Phe Ser Phe Phe Val Thr Leu Ala Leu

414 423 432 441 450 459

GTC GCT CGC TCT CTT GCA GCC ATC GGG CCG GTG GCG AGC CTC GTC GTC GCG AAC
Val Ala Arg Ser Leu Ala Ala Ile Gly Pro Val Ala Ser Leu Val Val Ala Asn

468 477 486 495 504 513

GCC CCC GTC TCG CCC GAC GGC TTC CTT CGG GAT GCC ATC GTG GTC AAC GGC GTG
Ala Pro Val Ser Pro Asp Gly Phe Leu Arg Asp Ala Ile Val Val Asn Gly Val

522 531 540 553 563 573

GTC CCT TCC CCG CTC ATC ACC GGG AAG AAG GTCGGCGTGT TCGTCGTCGT CCTACTCCT
Val Pro Ser Pro Leu Ile Thr Gly Lys Lys

FIG.2A

583 592 601 610 619 628

TGCTGACAGC GATCTACAG GGA GAC CGC GTC CAG CTC AAC GTC GTC GAC ACC TTG
Gly Asp Arg Phe Gln Leu Asn Val Val Asp Thr Leu

637 646 655 671 681 691

ACC AAC CAC AGC ATG CTC AAG TCC ACT AGT ATC GTAAGTGTGA CGATCCGAAT GTGACATCAA
Thr Asn His Ser MET Leu Lys Ser Thr Ser Ile

701 711 721 730 739 748

TCGGGGCTAA TTAACCGCGC ACAG CAC TGG CAC GGC TTC TTC CAG GCA GGC ACC AAC
His Trp His Gly Phe Phe Gln Ala Gly Thr Asn

757 766 775 784 793 802

TGG GCA GAA GGA CCC GCG TTC GTC AAC CAG TGC CCT ATT GCT TCC GGG CAT TCA
Trp Ala Glu Gly Pro Ala Phe Val Asn Gln Cys Pro Ile Ala Ser Gly His Ser

811 820 829 846 856

TTC CTG TAC GAC TTC CAT GTG CCC GAC CAG GCA G GTAAGCAGGA TTTTCTGGGG
Phe Leu Tyr Asp Phe His Val Pro Asp Gln Ala Gly

866 876 886 896 905 914

TCCCCGTGTG ATGCAATGTT CTCATGCTCC GACGTGATCG ACAG GG ACG TTC TGG TAC CAC
Thr Phe Trp Tyr His

923 932 941 950 959 968

AGT CAT CTG TCT ACG CAG TAC TGT GAC GGG CTG CGG GGG CCG TTC GTC GTG TAC
Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro Phe Val Val Tyr

977 986 995 1004 1013 1024

GAC CCC AAG GAC CCG CAC GCC AGC CGT TAC GAT GTT GAC AAT G GTACGTGCGC
Asp Pro Lys Asp Pro His Ala Ser Arg Tyr Asp Val Asp Asn Glu

FIG.2B

1034 1044 1054 1064 1075 1084

CACGGAGTAT ATCACACAGC ATGCGTTGAC GTCGGGCCAA CAGAG AGC ACG GTC ATC ACG
Ser Thr Val Ile Thr

1093 1102 1111 1120 1129 1141

TTG ACC GAC TGG TAC CAC ACC GCT GCC CGG CTC GGT CCC AAG TTC CC GTAAGCTCGC
Leu Thr Asp Trp Tyr His Thr Ala Ala Arg Leu Gly Pro Lys Phe Pro

1151 1161 1171 1181 1190 1199

AATGGCTTAG TGTTCACAGG TTCTTTGCTT ATGTTGCTTC GATAG A CTC GGC GCG GAC GCC
Leu Gly Ala Asp Ala

1208 1217 1226 1235 1244 1253

ACG CTC ATC AAC GGT CTG GGG CGG TCT GCC TCC ACT CCC ACC GCT GCG CTT GCC
Thr Leu Ile Asp Gly Leu Gly Arg Ser Ala Ser Thr Pro Thr Ala Ala Leu Ala

1262 1271 1280 1292 1302 1312

GTG ATC AAC GTC CAG CAC GGA AAG CG GTGAGCATTC TCTTGTATGC CATTTCAATG
Val Ile Asn Val Gln His Gly Lys Arg

1322 1332 1341 1351 1360 1369

CTTTGTGCTG ACCTATCGGA ACCGCGCAG C TAC CGC TTC CGT CTC GTT TCG ATC TCG
Tyr Arg Phe Arg Leu Val Ser Ile Ser

1378 1387 1396 1405 1414 1423

TGT GAC CCG AAC TAC ACG TTC AGC ATC GAC GGG CAC AAC CTG ACC GTC ATC GAG
Cys Asp Pro Asn Tyr Thr Phe Ser Ile Asp Gly His Asn Leu Thr Val Ile Glu

1432 1441 1450 1459 1468 1477

GTC GAC GGC ATC AAT AGC CAG CCT CTC CTT GTC GAC TCT ATC CAG ATC TTC GCC
Val Asp Gly Ile Asn Ser Gln Pro Leu Leu Val Asp Ser Ile Gln Ile Phe Ala

1486 1495 1508 1518 1528 1538

GCA CAG CGC TAC TCC TTC GTG GTAAGTCCTG GCTTGTCGAT GCTCCAAAGT GGCCTCACTC
Ala Gln Arg Tyr Ser Phe Val

FIG.2C

1548 1559 1568 1577 1586

ATATACTTTC GTTAG TTG AAT GCG AAT CAA ACG GTG GGC AAC TAC TGG GTT CGT
Leu Asn Ala Asn Gln Thr Val Gly Asn Tyr Trp Val Arg

1595 1604 1613 1622 1631 1640

GCG AAC CCG AAC TTC GGA ACG GTT GGG TTC GCC GGG GGG ATC AAC TCC GCC ATC
Ala Asn Pro Asn Phe Gly Thr Val Gly Phe Ala Gly Gly Ile Asn Ser Ala Ile

1649 1658 1667 1676 1685 1694

TTG CGC TAC CAG GGC GCA CCG GTC GCC GAG CCT ACC ACG ACC CAG ACG CCG TCG
Leu Arg Tyr Gln Gly Ala Pro Val Ala Glu Pro Thr Thr Thr Gln Thr Pro Ser

1703 1712 1721 1730 1739 1748 1761

GTG ATC CCG CTC ATC GAG ACG AAC TTG CAC CCG CTC GCG CGC ATG CCA GTG GTATGTCTCT
Val Ile Pro Leu Ile Glu Thr Asn Leu His Pro Leu Ala Arg MET Pro Val

1771 1781 1791 1801 1811 1821

TTTTCTGATC ATCTGAGTTG CCCGTTGTTG ACCGCATTAT GTGTTACTAT CTAG CCT GGC AGC
Pro Gly Ser

1830 1839 1848 1857 1866 1882

CCG ACA CCC GGG GGC GTC GAC AAG GCG CTC AAC CTC GCG TTT AAC TTC GTAAGTATCT
Pro Thr Pro Gly Gly Val Asp Lys Ala Leu Asn Leu Ala Phe Asn Phe

1892 1902 1912 1922 1931 1940

CTACTACTT GGCTGGAGGC TGGTCGCTGA TCATACGGTG CTTCAG AAC GGC ACC AAC TTC
Asn Gly Thr Asn Phe

1949 1958 1967 1976 1985 1994

TTC ATC AAC AAC GCG ACT TTC ACG CCG CCG ACC GTC CCG GTA CTC CTC CAG ATT
Phe Ile Asn Asn Ala Thr Phe Thr Pro Pro Thr Val Pro Val Leu Leu Gln Ile

FIG.2D

2003 2012 2021 2030 2039 2048

CTG AGC GGT GCG CAG ACC GCA CAA GAC CTG CTC CCC GCA GGC TCT GTC TAC CCG
Leu Ser Gly Ala Gln Thr Ala Gln Asp Leu Leu Pro Ala Gly Ser Val Tyr Pro

2057 2066 2075 2084 2093 2102

CTC CCG GCC CAC TCC ACC ATC GAG ATC ACG CTG CCC GCG ACC GCC TTG GCC CCG
Leu Pro Ala His Ser Thr Ile Glu Ile Thr Leu Pro Ala Thr Ala Leu Ala Pro

2111 2120 2129 2145 2155 2165

GGT GCA CCG CAC CCC TTC CAC CTG CAC GGT GTATGTTCCC CTGCCTTCCC TTCTTATCCC
Gly Ala Pro His Pro Phe His Leu His Gly

2175 2185 2195 2204 2213 2222

CGAACCAGTG CTCACGTCCG TCCCATCTAG CAC GCC TTC GCG GTC GTT CGC AGC GCG
His Ala Phe Ala Val Val Arg Ser Ala

2231 2240 2249 2258 2267 2276

GGG AGC ACC ACG TAT AAC TAC AAC GAC CCG ATC TTC CGC GAC GTC GTG AGC ACG
Gly Ser Thr Thr Tyr Asn Tyr Asn Asp Pro Ile Phe Arg Asp Val Val Ser Thr

2285 2294 2303 2312 2321 2330

GGC ACG CCC GCC GCG GGC GAC AAC GTC ACG ATC CGC TTC CAG ACG GAC AAC CCC
Gly Thr Pro Ala Ala Gly Asp Asn Val Thr Ile Arg Phe Gln Thr Asp Asn Pro

2339 2348 2357 2366 2375 2384

GGG CCG TGG TTC CTC CAC TGG CAC ATC GAC TTC CAC CTC GAC GCA GGC TTC GCG
Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Asp Ala Gly Phe Ala

2393 2402 2411 2420 2429 2438

ATC GTG TTC GCA GAG GAC GTT GCG GAC GTG AAG GCG GCG AAC CCG GTT CCG AAG
Ile Val Phe Ala Glu Asp Val Ala Asp Val Lys Ala Ala Asn Pro Val Pro Lys

FIG.2E

2447 2456 2465 2474 2483 2499

GCG TGG TCG GAC CTG TGC CCG ATC TAC GAG GGG CTG AGC GAG GCT AAC CAG TGAGCGGAGG
Ala Trp Ser Asp Leu Cys Pro Ile Tyr Asp Gly Leu Ser Glu Ala Asn Gln

2509 2519 2529 2539 2549 2559 2569

GCGTGGTGTG GAGCGTAAAG CTCGCGCGTC CACCTGGGGG GTTGAAGGTG TTCTGATTGA AATGGTCTTT

2579 2589 2599 2609 2619 2629 2639

GGGTTTATTT GTTGTTATTC TAACTCGGTT CTCTACGCAA GGACCGAGGA TTGTATAGGA TGAAGTAACT

2649 2659 2669 2679 2689

TTCCTAATGT ATTATGATAT CAATTGACGG AGGCATGGAC TCCGAAGTGT

FIG.2F

10 20 30 40 50 60 70
TTTCCCGACT AAACCAATCT CAGNCCGCTT CCTCCTAGGG AACCGAGCGA TGTGGCGGCC CTCTCTATCC

80 90 100 110 120 130 140
AAGCTGTCCA TAAGAAGACG TTCAAATGCC GCAGCAAGCG AGGAAATAAG CATCTAACAG TGTTTTTCCC

150 160 170 180 190 200 210
ATAGTCGCAT TTGCGCCGCC TGTCGGACCG ACGCCCCTAG AGCGCTTTGG GAAACGTCGC AAGTGGCGGG

220 230 240 250 260 270 280
TGTTATTCGT GTAGACGAGA CGGTATTTGT CTCATCATTC CCGTGCTTCA GGTTGACACA GCCCAAAGGT

290 300 310 320 330 340 350
CTATGTACGG CCCTTCACAT TCCCTGACAC ATTGACGCAA CCCTCGGTGC GCCTCCGACA GTGCCTCGGT

360 370 380 390 400 410 420
TGTAGTATCG GGACGCCCTA GGATGCAAGA TTGGAAGTCA CCAAGGCCCG AAGGGTATAA AATACCGAGA

430 440 450 460 470 480
GGTCCTACCA CTTCTGCATC TCCAGTCGCA GAGTTCCTCT CCCTTGCCAG CCACAGCTCG AG

491 500 509 518 527 536
>
ATG TCC TTC TCT AGC CTT CGC CGT GCC TTG GTC TTC CTG GGT GCT TGC AGC AGT
MET Ser Phe Ser Ser Leu Arg Arg Ala Leu Val Phe Leu Gly Ala Cys Ser Ser

545 554 563 572 581 590
GCG CTG GCC TCC ATC GGC CCA GTC ACT GAG CTC GAC ATC GTT AAC AAG GTC ATC
Ala Leu Ala Ser Ile Gly Pro Val Thr Glu Leu Asp Ile Val Asn Lys Val Ile

599 608 617 626 635 644
GCC CCG GAT GGC GTC GCT CGT GAT ACA GTC CTC GCC GGG GGC ACG TTC CCG GGC
Ala Pro Asp Gly Val Ala Arg Asp Thr Val Leu Ala Gly Gly Thr Phe Pro Gly

653 662 675 685 695 705
CCA CTC ATC ACA GGA AAG AAG GTATGCTAAG TAGTCCCGCC CCCATCATCC TGTGGCTGAC
Pro Leu Ile Thr Gly Lys Lys

FIG.3A

```
        715          726         735         744         753
GTTCGACGCC GCCAG GGT GAC AAC TTC CGC ATC AAC GTC GTC GAC AAG TTG GTT
                 Gly Asp Asn Phe Arg Ile Asn Val Val Asp Lys Leu Val
762         771         780         789         799         809         819
AAC CAG ACT ATG CTG ACA TCC ACC ACC ATT GTATGTCACT AGCTCTCGCT ATCTCGAGAC
Asn Gln Thr MET Leu Thr Ser Thr Thr Ile

        829         839         848         857         866         875
CCGCTGACCG ACAACATTTG CCGTAG CAC TGG CAC GGG ATG TTC CAG CAT ACG ACG
                             His Trp His Gly MET Phe Gln His Thr Thr

        884         893         902         911         920         929
AAC TGG GCG GAT GGT CCC GCC TTT GTG ACT CAA TGC CCT ATC ACC ACT GGT GAT
Asn Trp Ala Asp Gly Pro Ala Phe Val Thr Gln Cys Pro Ile Thr Thr Gly Asp

        938         947         956         965         976         986
GAT TTC CTG TAC AAC TTC CGC GTG CCC GAC CAG ACA G   GTACGCAAAG GGCAGCATGC
Asp Phe Leu Tyr Asn Phe Arg Val Pro Asp Gln Thr Gly

        996         1006        1016        1026        1035        1044
GTACTCAAAG ACATCTCTAA GCATTTGCTA CCTAG GA ACG TAC TGG TAC CAT AGC CAT
                                          Thr Tyr Trp Tyr His Ser His

        1053        1062        1071        1080        1089        1098
CTG GCC TTG CAG TAC TGT GAT GGG CTT CGC GGC CCC CTG GTG ATT TAC GAT CCC
Leu Ala Leu Gln Tyr Cys Asp Gly Leu Arg Gly Pro Leu Val Ile Tyr Asp Pro

        1107        1116        1125        1134        1145        1155
CAT GAT CCG CAG GCA TAC CTG TAT GAC GTC GAT GAC G   GTACGCAGCA CAGTTTCCCT
His Asp Pro Gln Ala Tyr Leu Tyr Asp Val Asp Asp Glu
```

FIG.3B

```
      1165       1175       1185          1198     1207
AAAACGGTTA ACTTCTAATT CTGTAAATAT CTTCATAG AG AGC ACC GTT ATC ACT CTG
                                             Ser Thr Val Ile Thr Leu

1216        1225        1234        1243        1252          1267
GCA GAC TGG TAC CAT ACC CCG GCG CCT CTG CTG CCG CCT GCC GC  GTACGCCTCC
Ala Asp Trp Tyr His Thr Pro Ala Pro Leu Leu Pro Pro Ala Ala

      1277       1287       1297       1307       1317        1328
ACACATCTGC ACAGCGTTCC GTATCTCATA CCCTTAAAGT TTATCGGACA G C ACT TTG ATT
                                                           Thr Leu Ile

     1337        1346        1355        1364        1373        1382
AAT GGC CTG GGT CGC TGC CCT GGC AAC CCC ACC GCC GAC CTA GCC GTC ATC GAA
Asp Gly Leu Gly Arg Trp Pro Gly Asn Pro Thr Ala Asp Leu Ala Val Ile Glu

     1391                   1409       1419       1429       1439       1449
GTC CAG CAC GGA AAG CG  GTATGTCATA GCTCGGTTAT CTATTCATAC TCGCGGCCTC GAAGCTAAAA
Val Gln His Gly Lys Arg

      1459         1470        1479        1488        1497
CCTTGTTCCA G C TAC CGG TTC CGA CTG GTC AGC ACC TCA TGC GAC CCC AAC TAC
               Tyr Arg Phe Arg Leu Val Ser Thr Ser Cys Asp Pro Asn Tyr

1506        1515        1524        1533        1542        1551
AAC TTC ACT ATC GAT GGC CAC ACC ATG ACA ATC ATC GAG GCG GAT GGG CAG AAC
Asn Phe Thr Ile Asp Gly His Thr MET Thr Ile Ile Glu Ala Asp Gly Gln Asn

1560        1569        1578        1587        1596        1605
ACC CAG CCA CAC CAA GTC GAC GGA CTT CAG ATC TTC GCG GCA CAG CGG TAC TCC
Thr Gln Pro His Gln Val Asp Gly Leu Gln Ile Phe Ala Ala Gln Arg Tyr Ser
```

FIG.3C

1614 1627 1637 1647 1657 1667

TTC GTT GTATGTTTTC CGCATTTCGG GAAAAGGAAT TGCGCTGACA GCTCGAGTGT GCGTAG
Phe Val

1676 1685 1694 1703 1712 1721

CTT AAC GCT AAC CAA GCG GTC AAC AAC TAC TGG ATC CGT GCG AAC CCT AAC CGT
Leu Asn Ala Asn Gln Ala Val Asn Asn Tyr Trp Ile Arg Ala Asn Pro Asn Arg

1730 1739 1748 1757 1766 1775

GCT AAC ACT ACG GGC TTC GCC AAC GGC ATC AAC TCC GCC ATC CTG CGC TAC AAG
Ala Asn Thr Thr Gly Phe Ala Asn Gly Ile Asn Ser Ala Ile Leu Arg Tyr Lys

1784 1793 1802 1811 1820 1829

GGG GCG CCG ATT AAG GAG CCT ACG ACG AAC CAG ACT ACC ATC CGG AAC TTT TTG
Gly Ala Pro Ile Lys Glu Pro Thr Thr Asn Gln Thr Thr Ile Arg Asn Phe Leu

1838 1847 1856 1865 1874 1884 1894

TGG GAG ACG GAC TTG CAC CCG CTC ACT GAC CCA CGT GCA GTAAGTTCTA CACAGTCACC
Trp Glu Thr Asp Leu His Pro Leu Thr Asp Pro Arg Ala

1904 1914 1924 1933 1942 1951

AACGGTGAGC TGTTGTCTGA TTGCACTGTG TTATAG CCT GGC CTT CCT TTC AAG GGG GGC
Pro Gly Leu Pro Phe Lys Gly Gly

1960 1969 1978 1987 1997 2007 2017

GTT GAC CAC GCT TTG AAC CTC AAC CTC ACT TTC GTACGTAGCG CCTCAGATAT CGAGTAGTCT
Val Asp His Ala Leu Asn Leu Asn Leu Thr Phe

2027 2037 2046 2055 2064 2073

ATCTCCTGAC CGATTGACAC AAT CCA TCG GAG TTC TTC ATC AAC GAT GCC CCT TTC
Asn Gly Ser Glu Phe Phe Ile Asn Asp Ala Pro Phe

FIG.3D

2082 2091 2100 2109 2118 2127

GTC CCT CCG ACT GTC CCG GTG CTA CTG CAG ATC CTG AAC GGA ACG CTC GAC GCG
Val Pro Pro Thr Val Pro Val leu Leu Gln Ile Leu Asn Gly Thr Leu Asp Ala

2136 2145 2154 2163 2172 2181

AAC GAC CTC CTG CCG CCC GGC AGC GTC TAC AAC CTT CCT CCG GAC TCC ACC ATC
Asn Asp Leu Leu Pro Pro Gly Ser Val Tyr Asn Leu Pro Pro Asp Ser Thr Ile

2190 2199 2208 2217 2226 2235

GAG CTG TCC ATT CCC GGA GGT GTG ACG GGT GGC CCG CAC CCA TTC CAT TTG CAC
Glu Leu Ser Ile Pro Gly Gly Val Thr Gly Gly Pro His Pro Phe His Leu His

2248 2258 2268 2278 2288 2297

GGG GTAATAATCT CTCTTTATAC TTTGGTCTCC CGATGCTGAC TTTCACTGCT CATCTTCAG
Gly

2306 2315 2324 2333 2342 2351

CAC GCT TTC TCC GTC GTG CGT AGC GCC GGC AGC ACC GAA TAC AAC TAC GCG AAC
His Ala Phe Ser Val Val Arg Ser Ala Gly Ser Thr Glu Tyr Asn Tyr Ala Asn

2360 2369 2378 2387 2396 2405

CCG GTG AAG CGC GAC ACG GTC AGC ATT GGT CTT GCG GGC GAC AAC GTC ACC GTG
Pro Val Lys Arg Asp Thr Val Ser Ile Gly Leu Ala Gly Asp Asn Val Thr Val

2414 2424 2434 2444 2454 2464

CGC TTC GTG GTATGTTTTA CAGCCTCTCT ATCTCCGTGG GCGTTCGGAA GTTGACTGGG CGCGTAG
Arg Phe Val

2474 2483 2492 2501 2510 2519

ACC GAC AAC CCC GGC CCG TGG TTC CTC CAC TGT CAC ATC GAC TTC CAT TTG CAA
Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Gln

FIG.3E

2528 2537 2546 2555 2564 2573

GCA GGC CTC GCC ATC GTG TTC GCG GAG GAC GCG CAG GAC ACG AAG CTT GTG AAC
Ala Gly Leu Ala Ile Val Phe Ala Glu Asp Ala Gln Asp Thr Lys Leu Val Asn

2582 2599 2609 2619 2629 2639

CCC GTC CCT G GTACGTCTTC TGGATGCATG CGCTCCGCAC AGTGACTCAT CTTTTGCAAC
Pro Val Pro Glu

2649 2658 2667 2676 2685

AG AG GAC TGG AAC AAG CTG TGC CCC ACC TTC GAT AAG GCG ATG AAC ATC ACG
Asp Trp Asn Lys Leu Cys Pro Thr Phe Asp Lys Ala MET Asn Ile Thr

2694 2704 2714 2724 2734 2744 2754

GTT TCAGCGATGC GTGGCGCTCA TGGTCATTTT CTTGGAATCT TTGCATAGGG CTGCAGCACG
Val

2764 2774 2784 2794 2804 2814 2824

CTGGATACTC TTTCCCTTAG CAGGATATTA TTTAATGACC CCTGCGTTTA GTGCTTAGTT AGCTTTACTA

2834 2844 2854 2864 2874 2884 2894

CTGGTTGTAA TGTACGCAGC ATGCGTAATT CGGATAATGC TATCAATGTG TATATTATGA CACGCGTCAT

2904 2914 2924 2934 2944 2954 2964

GCGCGATGCT TGAGTTGCAA GGTCGGTTTC CGATGCTCGA CATAAACGTT TCACTTACAT ACACATTGGG

2974 2984 2994 3004 3014 3024 3034

TCTAGAACTG GATCTATCCA TGTATACAAA AACTCCTCAT ACAGCTGACT GGGGCGCTCT AGAGCATGGG

3044 3054 3064 3074 3084 3094 3104

TCCGATTGAT CAGATGTCGC GAACACGAGC CTCCTGAGCT CGAGGACTCT GAGAAGCGGC GGTGCGTTCT

FIG.3F

10 20 30 40 50 60 70

GCGCGTTGGC CGATTCATTA ATGCAGCTGG CACGACAGGT TTCCCGACTG GAAAGCGGGC AGTGAGCGCA

80 90 100 110 120 130 140

ACGCAATTAA TGTGAGTTAG CTCACTCATT AGGCACCCCA GGCTTTACAC TTTATGCTTC CGGCTCGTAT

150 160 170 180 190 200 210

GTTGTGTGGA ATTGTGAGCG GATAACAATT TCACACAGGA AACACCTATG ACATGATTAC GAATTCCGAT

220 230 240 250 260 270 280

CGGCTTGCCC TCATTCCTCC ATGTTCCCCC GACCGAGCGG GCGCGTCAAT GGCCCGTTTG CGAACACATA

290 300 310 320 330 340 350

TGCAGGATAA ACAGTGCGAA ATATCAATGT GGCGGCGACA CAACCTCGCC GGCCGACACT CGACGCTGTT

360 370 380 390 400 410 420

GATCATGATC ATGTCTTGTG AGCATTCTAT ACGCAGCCTT GGAAATCTCA GGCGAATTTG TCTGAATTGC

430 440 450 460 470 480 490

GCTGGGAGGC TGGCAGCGCA GATCGGTGTG TCGGTGCAGT AGCCGACGCA GCACCTGGCG GAAGCCGACA

500 510 520 530 540 550 560

TCTCGGGTAC CACTTGATCT CCGCCAGATC ACTGCGGTTC CGCCATCGGC CGCGGGGCCC ATTCTGTGTG

570 580 590 600 610 620 630

TGCGCTGTAG CACTCTGCAT TCAGGCTCAA CGTATCCATG CTAGAGGACC GTCCAGCTGT TGGCGCACGA

640 650 660 670 680 690 700

TTCGCGCAGA AAGCTGTACA GGCAGATATA AGGATGTCCG TCCGTCAGAG ACTCGTCACT CACAAGCCTC

FIG.4A

710 720 730 740 750 760 770

TTTTCCTCTT CGCCTTTCCA GCCTCTTCCA ACGCCTGCCA TCGTCCTCTT AGTTCGCTCG TCCATTCTTT

780 790 799 808 817 826

CTGCGTAGTT AATC ATG GGC AGG TTC TCA TCT CTC TGC GCG CTC ACC GCC GTC ATC
MET Gly Arg Phe Ser Ser Leu Cys Ala Leu Thr Ala Val Ile

835 844 853 862 871 880

CAC TCT TTT GGT CGT GTC TCC GCC GCT ATC GGG CCT GTG ACC GAC CTC ACC ATC
His Ser Phe Gly Arg Val Ser Ala Ala Ile Gly Pro Val Thr Asp Leu Thr Ile

889 898 907 916 925 934

TCC AAT GGG GAC GTT TCT CCC GAC GGC TTC ACT CGT GCC GCA GTG CTT GCA AAC
Ser Asn Gly Asp Val Ser Pro Asp Gly Phe Thr Arg Ala Ala Val Leu Ala Asn

943 952 961 970 980 990

GGC GTC TTC CCG GGT CCT CTT ATC ACG GGA AAC AAG GTACGTGGCA TGCGTTCAGT
Gly Val Phe Pro Gly Pro Leu Ile Thr Gly Asn Lys

1000 1010 1020 1029 1038 1047

CTACACCCTA CAAGCCTTCT AACTCTTTTA CCACAG GGC GAC AAC TTC CAG ATC AAT GTT
Gly Asp Asn Phe Gln Ile Asn Val

1056 1065 1074 1083 1092 1105

ATC GAC AAC CTC TCT AAC GAG ACG ATG TTG AAG TCG ACC TCC ATC GTATGTGCTT
Ile Asp Asn Leu Ser Asn Glu Thr MET Leu Lys Ser Thr Ser Ile

1115 1125 1135 1145 1156 1165

CTACTGCTTC TTAGTCTTGG CAATGGCTCA AGGTCTCCTC CGCAG CAT TGG CAC GGC TTC
His Trp His Gly Phe

FIG.4B

1174 1183 1192 1201 1210 1219

TTC CAG AAG GGT ACT AAC TGG GCT GAT GGA GCT GCC TTC GTC AAC CAG TGC CCT
Phe Gln Lys gly thr Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro

1228 1237 1246 1235 1264

ATC GCG ACG GGG AAC TCT TTC CTT TAC GAC TTC ACC GCG ACG GAC CAA GCA G
Ile Ala Thr Gly Asn Ser Phe Leu Tyr Asp Phe Thr Ala Thr Asp Gln Ala Gly

1281 1291 1301 1311 1321 1331

GTCAGTGCCT GTGGCGCTTA TGTTTTCCCG TAATCAGCAG CTAACACTCC GCACCCACAG GC

1342 1351 1360 1369 1378 1387

ACC TTC TGG TAC CAC AGT CAC TTG TCT ACG CAG TAC TGC GAT GGT TTG CGG GGC
Thr Phe Trp Tyr His Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly

1396 1405 1414 1423 1432 1441

CCG ATG GTC GTA TAC GAC CCG AGT GAC CCG CAT GCG GAC CTT TAC GAC GTC GAC
Pro MET Val Val Tyr Asp Pro Ser Asp Pro His Ala Asp Leu Tyr Asp Val Asp

1450 1459 1468 1477 1486 1495

GAC GAG ACC ACG ATC ATC ACG CTC TCT GAT TGG TAT CAC ACC GCT GCT TCG CTC
Asp Glu Thr Thr Ile Ile Thr Leu Ser Asp Trp Tyr His Thr Ala Ala Ser Leu

1504 1519 1529 1539 1549 1559

GGT GCT GCC TTC CC GTAAGTTTAC CCCAGCGCAC GGAGTTAAGA CCGATCTAA CTGTAATACG
Gly Ala Ala Phe Pro

1568 1577 1586 1604 1614

TTCAG G ATT GGC TCG GAC TCT ACC CTG ATT AAC GG GTTGGCCGCT TCGCGGGTGG
Ile Gly Ser Asp Ser Thr Leu Ile Asn Gly

FIG.4C

1624 1633 1642 1651 1669

TGACAG C ACT GAC CTT GCG GTT ATC ACT GTC GAG CAG GGC AAG CG GTTAGTGATA
Thr Asp Leu Ala Val Ile Thr Val Glu Gln Gly Lys Arg

1679 1689 1699 1709 1719 1728

CCCTCTACAG TTGACACTGT GCCATTGCTG ACAGTACTCT CAG C TAC CGT ATG CGT CTT
Tyr Arg MET Arg Leu

1737 1746 1755 1764 1773 1782

CTC TCG CTG TCT TGC GAC CCC AAC TAT GTC TTC TCC ATT GAC GGC CAC AAC ATG
Leu Ser Leu Ser Cys Asp Pro Asn Tyr Val Phe Ser Ile Asp Gly His Asn MET

1791 1800 1809 1818 1827 1836

ACC ATC ATC GAG GCC GAC GCC GTC AAC CAC GAG CCC CTC ACG GTT GAC TCC ATC
Thr Ile Ile Gln Ala Asp Ala Val Asn His Glu Pro Leu Thr Val Asp Ser Ile

1845 1854 1863 1879 1889 1899

CAG ATC TAC GCC GGC CAA CGT TAC TCC TTC GTC GTACGTATTC CGAACAGCCA TGATCACGCC
Gln Ile Tyr Ala Gly Gln Arg Tyr Ser Phe Val

1909 1919 1928 1937 1946 1955

AAGCCCGATG CTAACGCGCC TACCCTCAG CTT ACC GCT GAC CAG GAC ATC GAC AAC TAC
Leu Thr Ala Asp Gln Asp Ile Asp Asn Tyr

1964 1973 1982 1991 2000 2009

TTC ATC CGT GCC CTG CCC AGC GCC GGT ACC ACC TCG TTC GAC GGC GGC ATC AAC
Phe Ile Arg Ala Leu Pro Ser Ala Gly Thr Thr Ser Phe Asp Gly Gly Ile Asn

2018 2027 2036 2045 2054 2063

TCG GCT ATC CTG CGC TAC TCT GGT GCC TCC GAG GTT GAC CCG ACG ACC ACG GAG
Ser Ala Ile Leu Arg Tyr Ser Gly Ala Ser Glu Val Asp Pro Thr Thr Thr Glu

FIG.4D

2072 2081 2090 2099 2108 2117

ACC ACG AGC GTC CTC CCC CTC GAC GAG GCG AAC CTC GTG CCC CTT GAC AGC CCC
Thr Thr Ser Val Leu Pro Leu Asp Glu Ala Asn Leu Val Pro Leu Asp Ser Pro

2126 2136 2146 2156 2166 2176

GCT GCT GTACGTCGTA TTCTGCGCTT GCAAGGATCG CACATACTAA CATGCTCTTG TAG CCC
Ala Ala Pro

2185 2194 2203 2212 2221 2230

GGT GAC CCC AAC ATT GGC GGT GTC GAC TAC GCG CTG AAC TTG GAC TTC AAC TTC
Gly Asp Pro Asn Ile Gly Gly Val Asp Tyr Ala Leu Asn Leu Asp Phe Asn Phe

2239 2248 2257 2266 2275 2284

GAT GGC ACC AAC TTC TTC ATC AAC GAC GTC TCC TTC GTG TCC CCC ACG GTC CCT
Asp Gly Thr Asn Phe Phe Ile Asn Asp Val Ser Phe Val Ser Pro Thr Val Pro

2293 2302 2311 2320 2329 2338

GTC CTC CTC CAG ATT CTT AGC GGC ACC ACC TCC GCG GCC GAC CTT CTC CCC AGC
Val Leu Leu Gln Ile Leu Ser Gly Thr Thr Ser Ala Ala Asp Leu Leu Pro Ser

2347 2356 2365 2374 2383 2392

GGT AGT CTC TTC GCG GTC CCG TCC AAC TCG ACG ATC GAG ATC TCG TTC CCC ATC
Gly Ser Leu Phe Ala Val Pro Ser Asn Ser Thr Ile Glu Ile Ser Phe Pro Ile

2401 2410 2419 2428 2437 2446 2456

ACC GCG ACG AAC GCT CCC GGC GCG CCG CAT CCC TTC CAC TTG CAC GGT GTACGTGTCC
Thr Ala Thr Asn Ala Pro Gly Ala Pro His Pro Phe His Leu His Gly

2466 2476 2486 2496 2506 2515

CATCTCATAT GCTACGGAGC TCCACGCTGA CCGCCCTATA G CAC ACC TTC TCT ATC GTT
His Thr Phe Ser Ile Val

FIG.4E

2524 2533 2542 2551 2560 2569

CGT ACC GCC GGC AGC ACG GAT ACG AAC TTC GTC AAC CCC GTC CGC CGC GAC GTC
Arg Thr Ala Gly Ser Thr Asp Thr Asn Phe Val Asn Pro Val Arg Arg Asp Val

2578 2587 2596 2605 2614 2624

GTG AAC ACC GGT ACC GTC GGC GAC AAC GTC ACC ATC CGC TTC ACG GTACGCAGCA
Val Asn Thr Gly Thr Val Gly Asp Asn Val Thr Ile Arg Phe Thr

2634 2644 2654 2664 2673 2682

CTCTCCTAAC ATTCCCACTG CGCGATCACT GACTCCTCGC CCACAG ACT GAC AAC CCC GGC
Thr Asp Asn Pro Gly

2691 2700 2709 2718 2727 2736

CCC TGG TTC CTC CAC TGC CAC ATC GAC TTC CAC TTG GAG GCC GGT TTC GCC ATC
Pro Trp Phe Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile

2745 2754 2763 2772 2781 2798

GTC TTC AGC GAG GAC ACC GCC GAC GTC TCG AAC ACG ACC ACG CCC TCG A GTACGTTGTG
Val Phe Ser Glu Asp Thr Ala Asp Val Ser Asn Thr Thr Thr Pro Ser Thr

2808 2818 2828 2838 2850 2859

CTCCCGTGCC CATCTCCGCG CGCCTGACTA ACGAGCACCC CTTACAG CT GCT TGG GAA GAT
Ala Trp Glu Asp

2868 2877 2886 2895 2908 2918

CTG TGC CCC ACG TAC AAC GCT CTT GAC TCA TCC GAC CTC TAATCGGTTC AAAGGGTCGC
Leu Cys Pro Thr Tyr Asn Ala Leu Asp Ser Ser Asp Leu

2928 2938 2948 2958 2968 2978 2988

TCGCTACCTT AGTAGGTAGA CTTATGCACC GGACATTATC TACAATGGAC TTTAATTTGG GTTAACGGCC

2998 3008 3018 3028 2038 3048 3058

GTTATACATA CGCGCACGTA GTATAAAGGT TCTCTGGATT GGTCGGACCT ACAGACTGCA ATTTTCGTGA

3068 3078 3088 3098

CCTATCAACT GTATATTGAA GCACGCAGT GAATGGAAAT AGAGACA

FIG.4F

10 20 30 40 50 60 70

CTCATAACTC TTCGCTTCTA GCATGGGGGC TGCGCACACC TGACAGACCC TTCGGGAGGC GAACTCGAAT

80 90 100 110 120 130 140

GCAGCGTACT CTATCNCACC TCCAGGAAAG GTAGGGATGG ACNCCGTGCA CCAACAACTG TCTCTCCACC

150 160 170 180 190 200 210

AGCAACCATC CCTTGGATAT GTCTCCACAC ACCCGGTGTC TACAAGCGGG GATCTGTGCT GGTGAAGTGC

220 230 240 250 260 270 280

TGTCTCCGGA GCGGCGGCGG CGAGCGACCA GAACCCGAAC CAGTGCTAGT GCCCGACACC CGCGAGACAA

290 300 310 320 330 340 350

TTGTGCAGGG TGAGTTATAT TCTTCGTGAG ACGGCGCTGC GCGTCGGCAC TGAAAGCGTC GCAGTTAGGT

360 370 380 390 400 410 420

GATGCAGCGG TCCGCGCTAT TTTTGACGTC TGGCAGCTAT CCTAAGCCGC GCCTCCATAC ACCCCAGGCG

430 440 450 460 470 480 490

CTCTCGTTTG CTATAGGTAT AAATCCCTCA GCTTCAGAGC GTCGATCCTC ATCCCACACG ACACCCGTTT

500 510 520 530 540 550

CAGTCTTCTC GTAGCGCATT CCCTAGCCGC CCAGCCTCCG CTTTCGTTTT CAAC ATG GGC AAG
MET Gly Lys

559 568 577 586 595 604

TAT CAC TCT TTT GTG AAC GTC GTC GCC CTT AGT CTT TCT TTG AGC GGT CGT GTG
Tyr His Ser Phe Val Asn Val Val Ala Leu Ser Leu Ser Leu Ser Gly Arg Val

613 622 631 640 649 658

TTC GGC GCC ATT GGG CCC GTC ACC GAC TTG ACT ATC TCT AAC GCC GAT GTT ACG
Phe Gly Ala Ile Gly Pro Val Thr Asp Leu Thr Ile Ser Asn Ala Asp Val Thr

FIG.5A

```
   667       676       685       694       703       712
CCT GAC GGC ATT ACT CGT GCT GCT GTC CTC GCG GGC GGC GTT TTC CCC GGG CCC
Pro Asp Gly Ile Thr Arg Ala Ala Val Leu Ala Gly Gly Val Phe Pro Gly Pro

   721       730          743        753        763        773        783
CTC ATT ACC GGC AAC AAG GTGAGCCGCG AAACCTTCTA CTAGCGCGCT CGTACGGTGC ACCGTTACTG
Leu Ile Thr Gly Asn Lys

     793        803          814       823       832       841
AAGCCACACT TTGCGCTGTC AACAG GGG GAT GAA TTC CAG ATC AAT GTC ATC GAC AAC
                            Gly Asp Glu Phe Gln Ile Asn Val Ile Asp Asn

     850       859       868       877       887        897
CTG ACC AAC GAG ACC ATG TTG AAG TCG ACC ACA ATC GTAAGGTGCT TGCTCCCATA
Leu Thr Asn Glu Thr MET Leu Lys Ser Thr Thr Ile

     907        917        927          938       947       956
ATTAAGCCCG TCGCTGACTC GAAGTTTATC TGTAG CAC TGG CAT GGT ATC TTC CAG GCC
                                      His Trp His Gly Ile Phe Gln Ala

     965       974       983       992       1001      1010
GGC ACC AAC TGG GCA GAC GGC GCG GCC TTC GTG AAC CAG TGC CCT ATC GCC ACG
Gly Thr Asn Trp Ala Asp Gly Ala Ala Phe Val Asn Gln Cys Pro Ile Ala Thr

     1019      1028      1037      1046                1063
GGA AAC TCG TTC TTG TAC GAC TTC ACC GTT CCT GAT CAA GCC G   GTACGTTTAT
Gly Asn Ser Phe Leu Tyr Asp Phe Thr Val Pro Asp Gln Ala Gly

     1073       1083       1093       1103       1112      1121
ACACTTCCCT TTCTGCGGCA TACTCTGACG CGCCGCTGGA TCAG GC ACC TTC TGG TAC CAC
                                                     Thr Phe Trp Tyr His
```

FIG.5B

1130 1139 1148 1157 1166 1175

AGC CAC CTG TCC ACC CAG TAC TGT GAC GGC CTG CGC GGT CCT CTT GTG GTC TAC
Ser His Leu Ser Thr Gln Tyr Cys Asp Gly Leu Arg Gly Pro Leu Val Val Tyr

1184 1193 1202 1211 1220 1231

GAC CCC GAC GAT CCC AAC GCG TCT CTT TAC GAC GTC GAT GAC G GTAAGCAGGC
Asp Pro Asp Asp Pro Asn Ala Ser Leu Tyr Asp Val Asp Asp Asp

1241 1251 1261 1271 1281 1290
TACTTGTGGA CTTGTATGGA TGTATCTCAC GCTCCCCTAC AG AT ACT ACG GTT ATT ACG
Thr Thr Val Ile Thr

1299 1308 1317 1326 1335 1347

CTT GCG GAC TGG TAC CAC ACT GCG GCG AAG CTG GGC CCT GCC TTC CC GTGAGTCTAC
Leu Ala Asp Trp Tyr His Thr Ala Ala Lys Leu Gly Pro Ala Phe Pro

1357 1367 1377 1387 1397 1408

TCTTCCTCGT GTGTTAACAT AGGTGACGGC CGCTGATACG AGAGCTACCA G C GCG GGT CCG
Ala Gly Pro

1417 1426 1435 1444 1453 1462

GAT AGC GTC TTG ATC AAT GGT CTT GGT CGG TTC TCC GGC GAT GGT GGA GGA GCG
Asp Ser Val Leu Ile Asn Gly Leu Gly Arg Phe Ser Gly Asp Gly Gly Gly Ala

1471 1480 1489 1498 1510 1520

ACA AAC CTC ACC GTG ATC ACC GTC ACG CAA GGC AAA CG GTGAGTCCGC CCTGAGCTGG
Thr Asn Leu Thr Val Ile Thr Val Thr Gln Gly Lys Arg

1530 1540 1550 1561 1570 1579

CCTCAATAGC GATATTGACG AGTCCATGCC CTCCCAG G TAC CGC TTC CGC CTT GTG TCG
Tyr Arg Phe Arg Leu Val Ser

FIG.5C

1588 1597 1606 1615 1624 1633

ATC TCG TGC GAC CCC AAC TTC ACG TTC TCG ATC GAC GGG CAC AAC ATG ACC ATC
Ile Ser Cys Asp Pro Asn Phe Thr Phe Ser Ile Asp Gly His Asn MET Thr Ile

1642 1651 1660 1669 1678 1687

ATC GAG GTG GAC GGT GTC AAC CAC GAG GCC TTG GAC GTC GAC TCC ATT CAG ATT
Ile Glu Val Asp Gly Val Asn His Glu Ala Leu Asp Val Asp Ser Ile Gln Ile

1696 1705 1714 1724 1734 1744

TTT GCG GGG CAG CGG TAC TCC TTC ATC GTACGTTCCC TTGCCCTCGT GCTATATCCG
Phe Ala Gly Gln Arg Tyr Ser Phe Ile

1754 1764 1774 1785 1794 1803

CCCGTCTGCT CACAGAGGCT TCTATATCGC AG CTC AAC GCC AAC CAG TCC ATC GAC AAC
Leu Asn Ala Asn Gln Ser Ile Asp Asn

1812 1821 1830 1839 1848 1857

TAC TGG ATC CGC GCG ATC CCC AAC ACC GGT ACC ACC GAC ACC ACG GGC GGC GTG
Tyr Trp Ile Arg Ala Ile Pro Asn Thr Gly Thr Thr Asp Thr Thr Gly Gly Val

1866 1875 1884 1893 1902 1911

AAC TCT GCT ATT CTT CGC TAC GAC ACC GCA GAA GAT ATC GAG CCT ACG ACC AAC
Asn Ser Ala Ile Leu Arg Tyr Asp Thr Ala Glu Asp Ile Glu Pro Thr Thr Asn

1920 1929 1938 1947 1956 1965

GCG ACC ACC TCC GTC ATC CCT CTC ACC GAG ACG GAT CTG GTG CCG CTC GAC AAC
Ala Thr Thr Ser Val Ile Pro Leu Thr Glu Thr Asp Leu Val Pro Leu Asp Asn

1974 1983 1992 2001 2010 2019

CCT GCG GCT CCC GGT GAC CCC CAG GTC GGC GGT GTT GAC CTG GCT ATG AGT CTC
Pro Ala Ala Pro Gly Asp Pro Gln Val Gly Gly Val Asp Leu Ala MET Ser Leu

FIG.5D

2028 2041 2051 2061 2071 2081

GAC TTC TCC TTC CTGAGTCCCA CAGGACTCCG CGCCATTTCC CTTATTTACG CAGGAGTATT
Asp Phe Ser Phe

2090 2099 2108 2117 2126 2135

GTTCAG AAC GGT TCC AAC TTC TTT ATC AAC AAC GAG ACC TTC GTC CCG CCC ACA
Asn Gly Ser Asn Phe Phe Ile Asn Asn Glu Thr Phe Val Pro Pro Thr

2144 2153 2162 2171 2180 2189

GTT CCC GTG CTC CTG CAG ATT TTG AGT GGT GCG CAG GAC GCG GCG AGC CTG CTC
Val Pro Val Leu Leu Gln Ile Leu Ser Gly Ala Gln Asp Ala Ala Ser Leu Leu

2198 2207 2216 2225 2234 2243

CCC AAC GGG AGT GTC TAC ACA CTC CCT TCG AAC TCG ACC ATT GAG ATC TCG TTC
Pro Asn Gly Ser Val Tyr Thr Leu Pro Ser Asn Ser Thr Ile Glu Ile Ser Phe

2252 2261 2270 2279 2288 2297

CCC ATC ATC ACC ACC GAC GGT GTT CTG AAC GCG CCC GGT GCT CCG CAC CCG TTC
Pro Ile Ile Thr Thr Asp Gly Val Leu Asn Ala Pro Gly Ala Pro His Pro Phe

2306 2319 2329 2339 2349 2359

CAT CTC CAC GGC GTAAGTCCTT GCTTTCCTCA GTGCCTCGCT TCCACGACGT CCACTGATCC
His Leu His Gly

2369 2380 2389 2398 2407 2416

CACACATCCC ATGTGCAG CAC ACC TTC TCG GTG GTG CGC AGC GCC GGG AGC TCG ACC
His Thr Phe Ser Val Val Arg Ser Ala Gly Ser Ser Thr

2425 2434 2443 2452 2461 2470

TTC AAC TAC GCC AAC CCA GTC CGC CGG GAC ACC GTC AGT ACT GGT AAC TCT GGC
Phe Asn Tyr Ala Asn Pro Val Arg Arg Asp Thr Val Ser Thr Gly Asn Ser Gly

FIG.5E

```
2479      2488                2504       2514       2524       2534
GAC AAC GTC ACT ATC CGC TTC ACG GTACGTCTTC TCCGGAGCCC TCCCACCCGT GTGTCCGCTG
Asp Asn Val Thr Ile Arg Phe Thr

     2544       2554       2564        2574        2583        2592
AGCGCTGAAC ACCGCCCACC GTGCTGCTGC TGCGCAG ACC GAC AAC CCA GGC CCG TGG
                                         Thr Asp Asn Pro Gly Pro Trp

     2601        2610        2619        2628        2637        2646
TTC CTC CAC TGC CAC ATC GAC TTC CAC CTG GAG GCC GGC TTC GCC ATC GTC TGG
Phe Leu His Cys His Ile Asp Phe His Leu Glu Ala Gly Phe Ala Ile Val Trp

     2655        2664        2673        2682                     2699
GGG GAG GAC ACT GCG GAC ACC GCG TCC GCG AAT CCC GTT CCT A   GTACGTCGTG
Gly Glu Asp Thr Ala Asp Thr Ala Ser Ala Asn Pro Val Pro Thr

     2709       2710       2729       2739       2749        2759
CCTGCTGAGC TCTTTGTGCC CCAACAGGGT GCTGATCGTC CCTTCCTCCG TGCAG CG GCG TGG
                                                                Ala Trp

   2768        2777        2786        2795        2804            2817
AGC GAT TTG TGC CCC ACT TAC GAT GCT TTG GAC TCG TCC GAC CTC TGATCGACAA
Ser Asp Leu Cys Pro Thr Tyr Asp Ala Leu Asp Ser Ser Asp Leu

     2827       2837       2847       2857       2867       2877       2887
GGCATGAAGG CTGAAGCAGC TGCGGTCAAT TCTCGAACAC ACTTTACTCG AACATTCATT TTTCTTTGGC

     2897       2907       2917
TCGGGATCGG AACAAATCAT GGGGGGGCCG GACCGTCT
```

FIG.5F

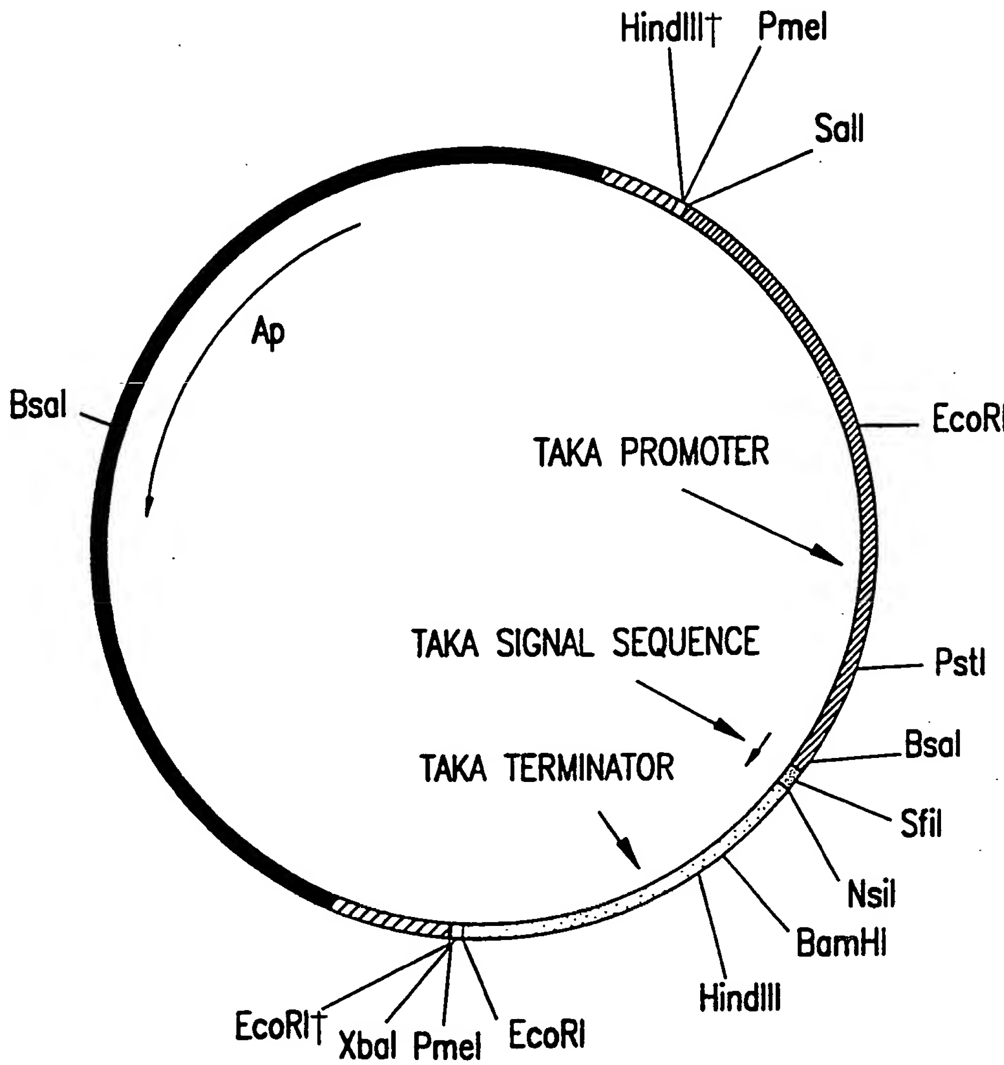

HindIII† PmeI
SalI
Ap
BsaI
EcoRI
TAKA PROMOTER
TAKA SIGNAL SEQUENCE
PstI
BsaI
TAKA TERMINATOR
SfiI
NsiI
BamHI
HindIII
EcoRI† XbaI PmeI EcoRI

FIG.6

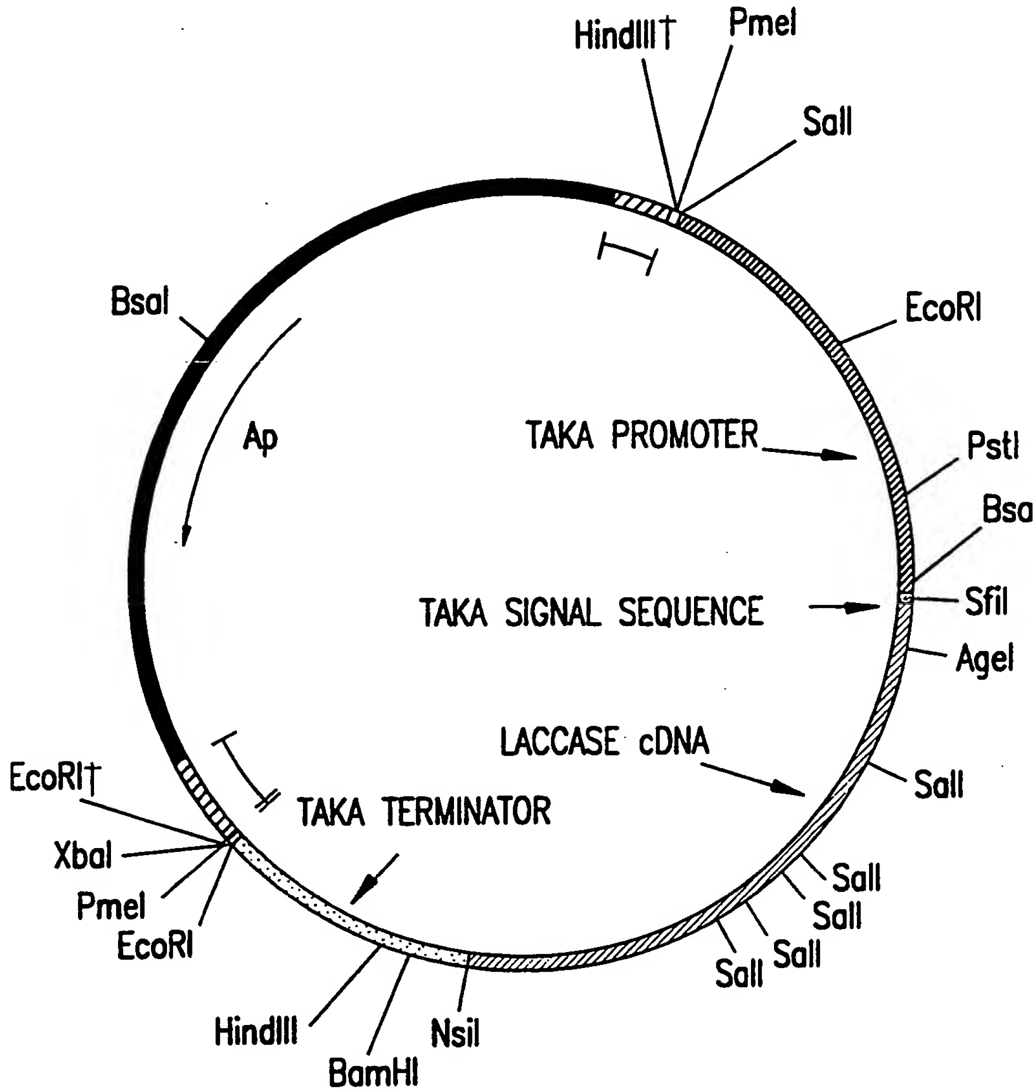
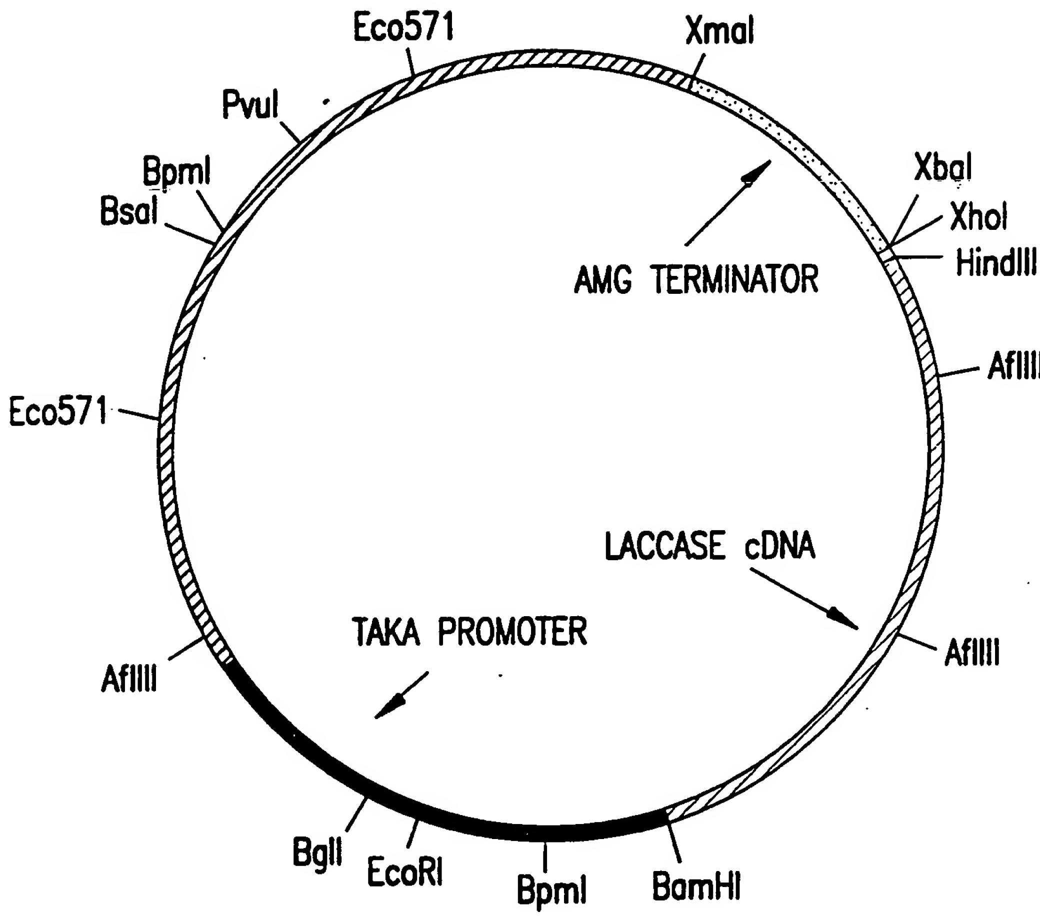

HindIII†
PmeI
SalI
EcoRI
BsaI
Ap
TAKA PROMOTER
PstI
BsaI
TAKA SIGNAL SEQUENCE
SfiI
AgeI
LACCASE cDNA
SalI
EcoRI†
TAKA TERMINATOR
XbaI
PmeI
EcoRI
SalI
SalI
SalI
SalI
HindIII
BamHI
NsiI

FIG.7



Eco571
XmaI
PvuI
BpmI
BsaI
XbaI
XhoI
HindIII
AMG TERMINATOR
AflIII
Eco571
LACCASE cDNA
TAKA PROMOTER
AflIII
AflIII
BglI
EcoRI
BpmI
BamHI

FIG.8

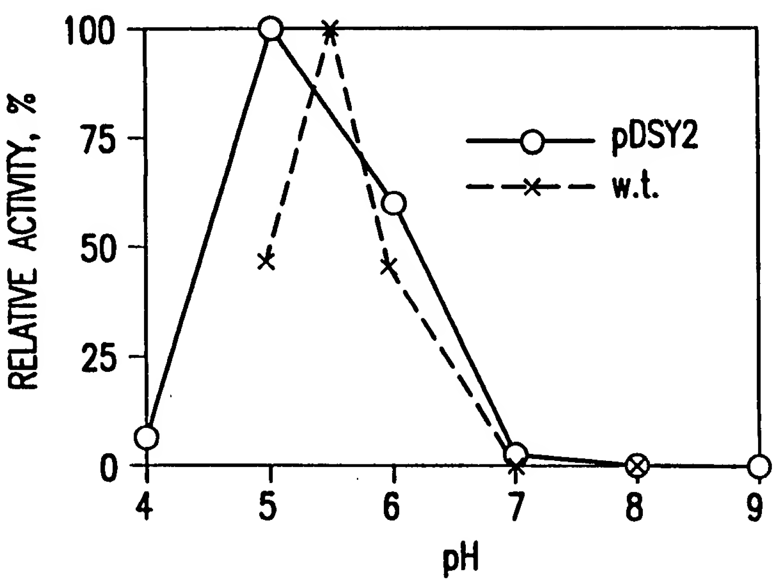

RELATIVE ACTIVITY, %
100
75
50
25
0
4
5
6
7
8
9
pH
pDSY2
w.t.

FIG.9A

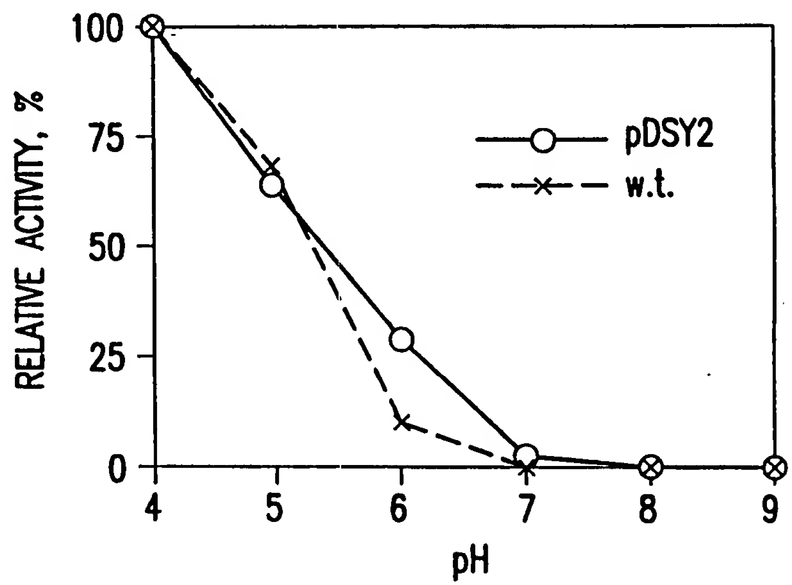

RELATIVE ACTIVITY, %
100
75
50
25
0
4
5
6
7
8
9
pH
pDSY2
w.t.

FIG.9B

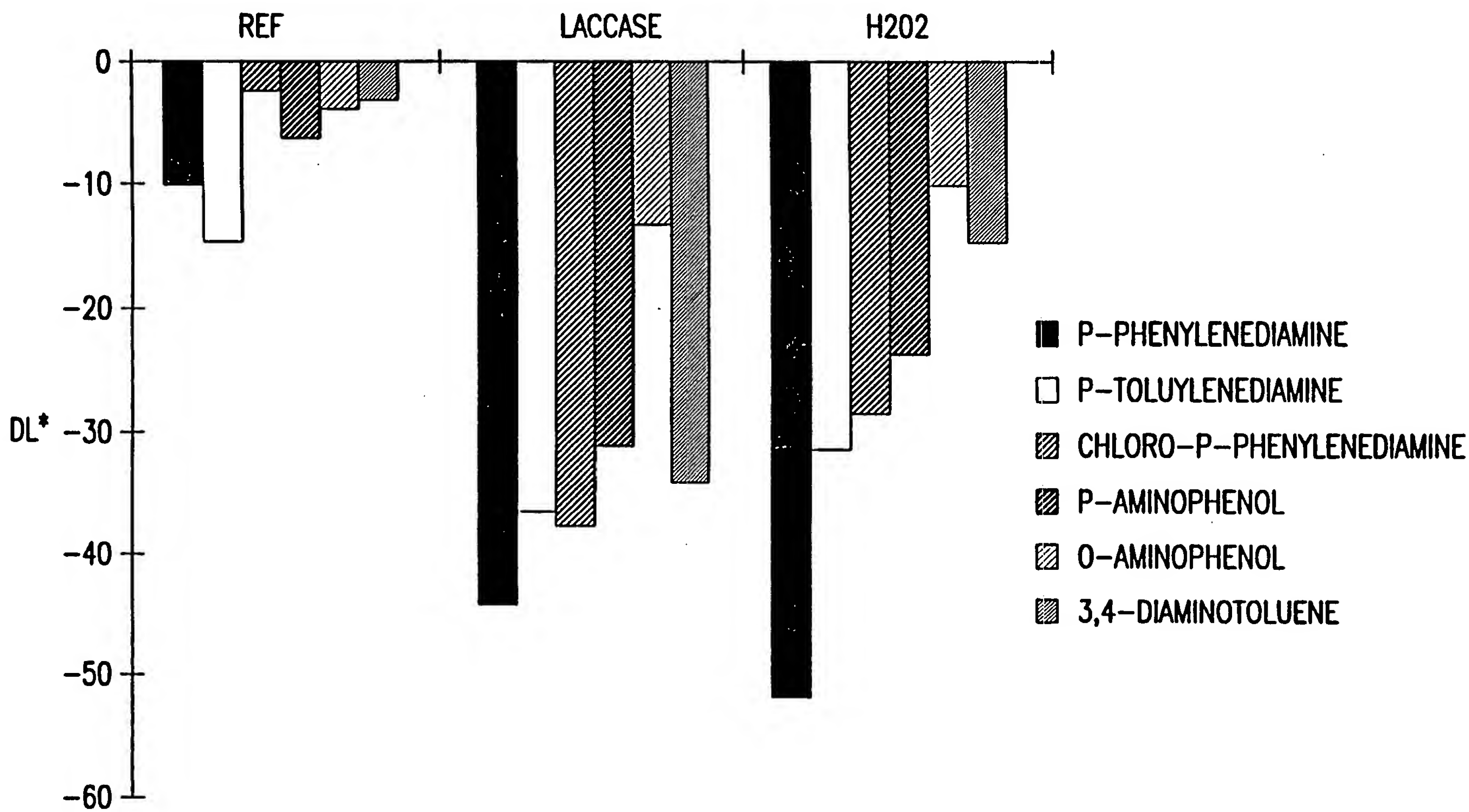
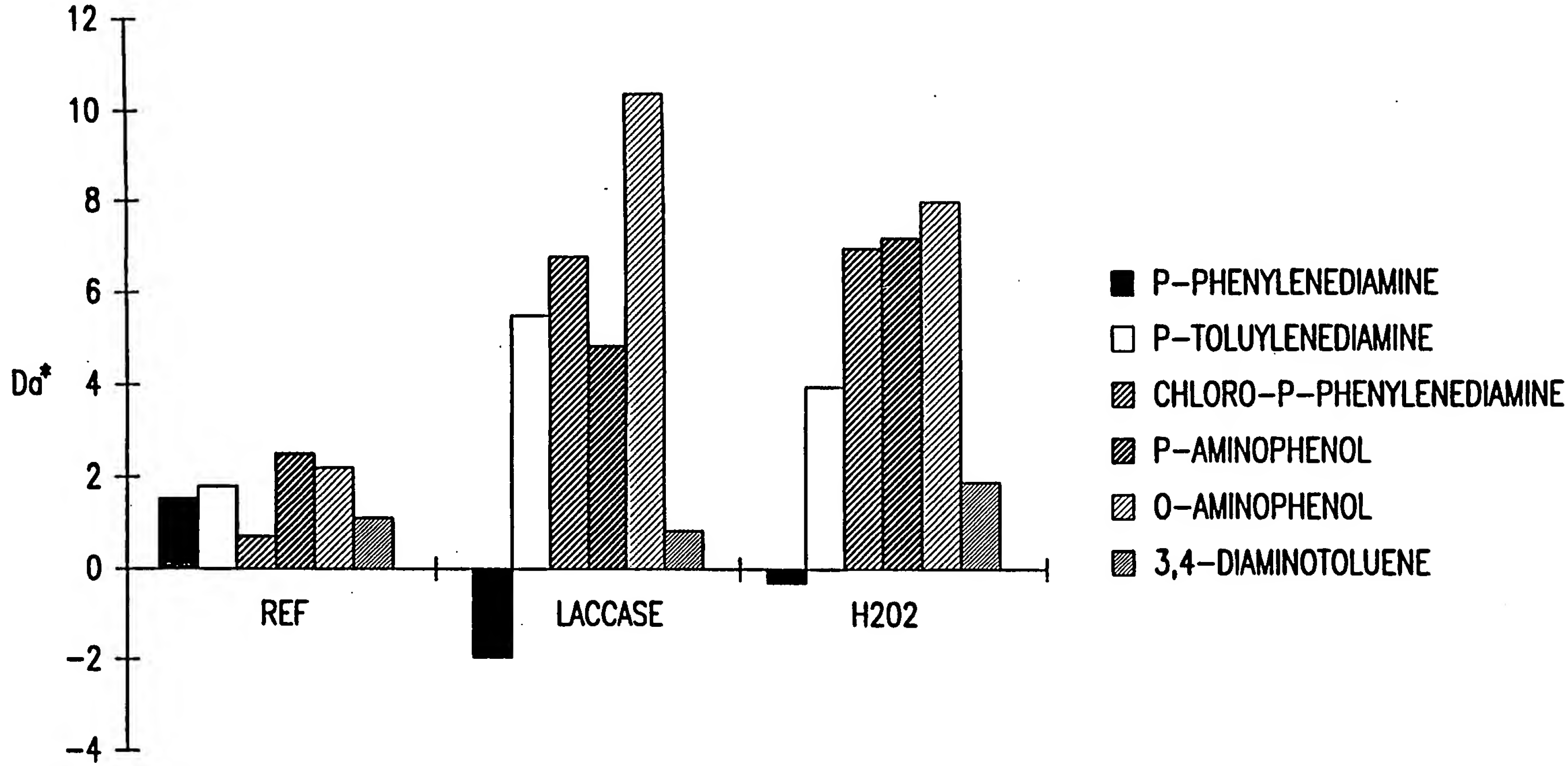

REF
LACCASE
H202
DL*
0
-10
-20
-30
-40
-50
-60
P-PHENYLENEDIAMINE
P-TOLUYLENEDIAMINE
CHLORO-P-PHENYLENEDIAMINE
P-AMINOPHENOL
O-AMINOPHENOL
3,4-DIAMINOTOLUENE

FIG.10



Da*
12
10
8
6
4
2
0
-2
-4
REF
LACCASE
H202
P-PHENYLENEDIAMINE
P-TOLUYLENEDIAMINE
CHLORO-P-PHENYLENEDIAMINE
P-AMINOPHENOL
O-AMINOPHENOL
3,4-DIAMINOTOLUENE

FIG.11

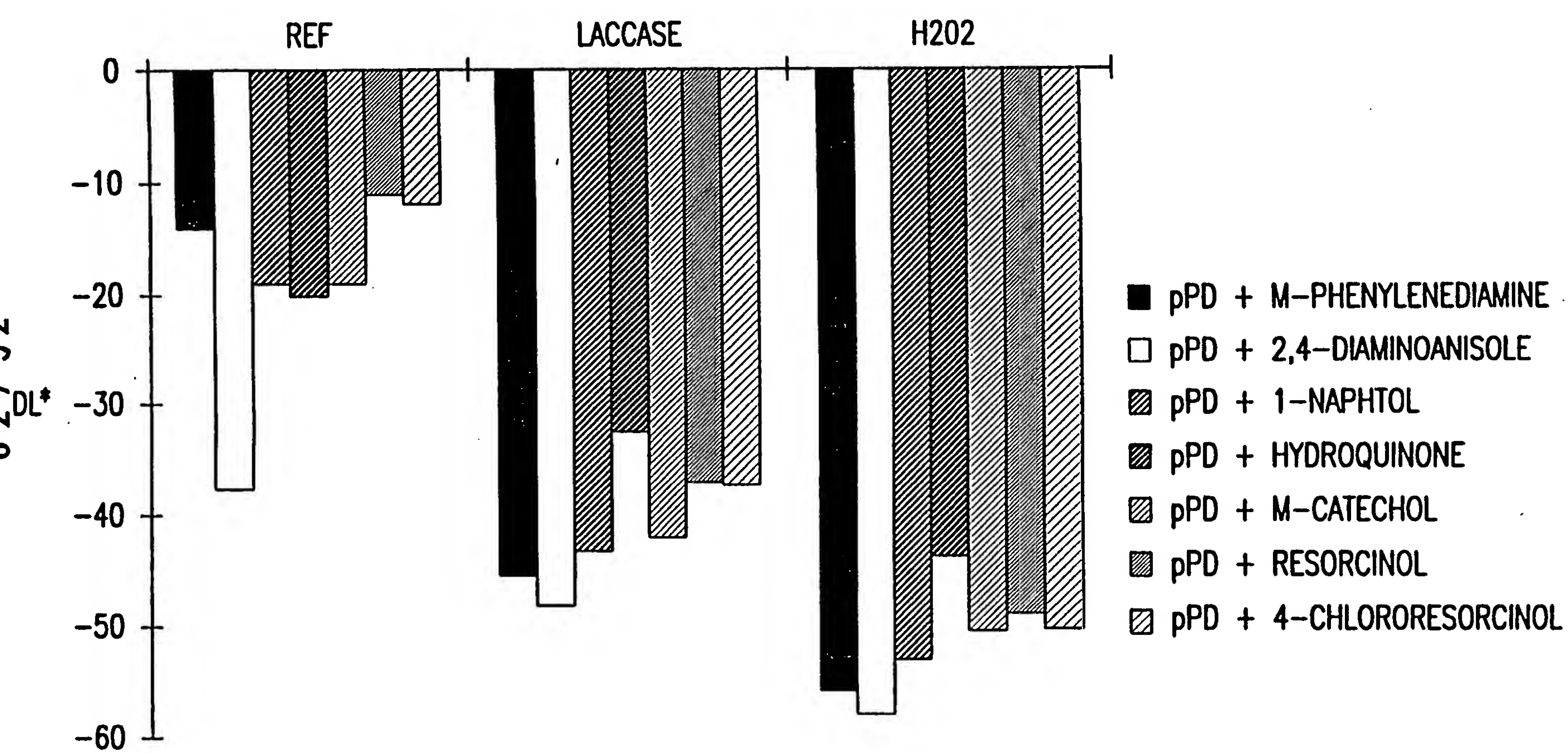

REF
LACCASE
H202
0
-10
-20
-30
-40
-50
-60
DL*
pPD + M-PHENYLENEDIAMINE
pPD + 2,4-DIAMINOANISOLE
pPD + 1-NAPHTOL
pPD + HYDROQUINONE
pPD + M-CATECHOL
pPD + RESORCINOL
pPD + 4-CHLORORESORCINOL

FIG.12

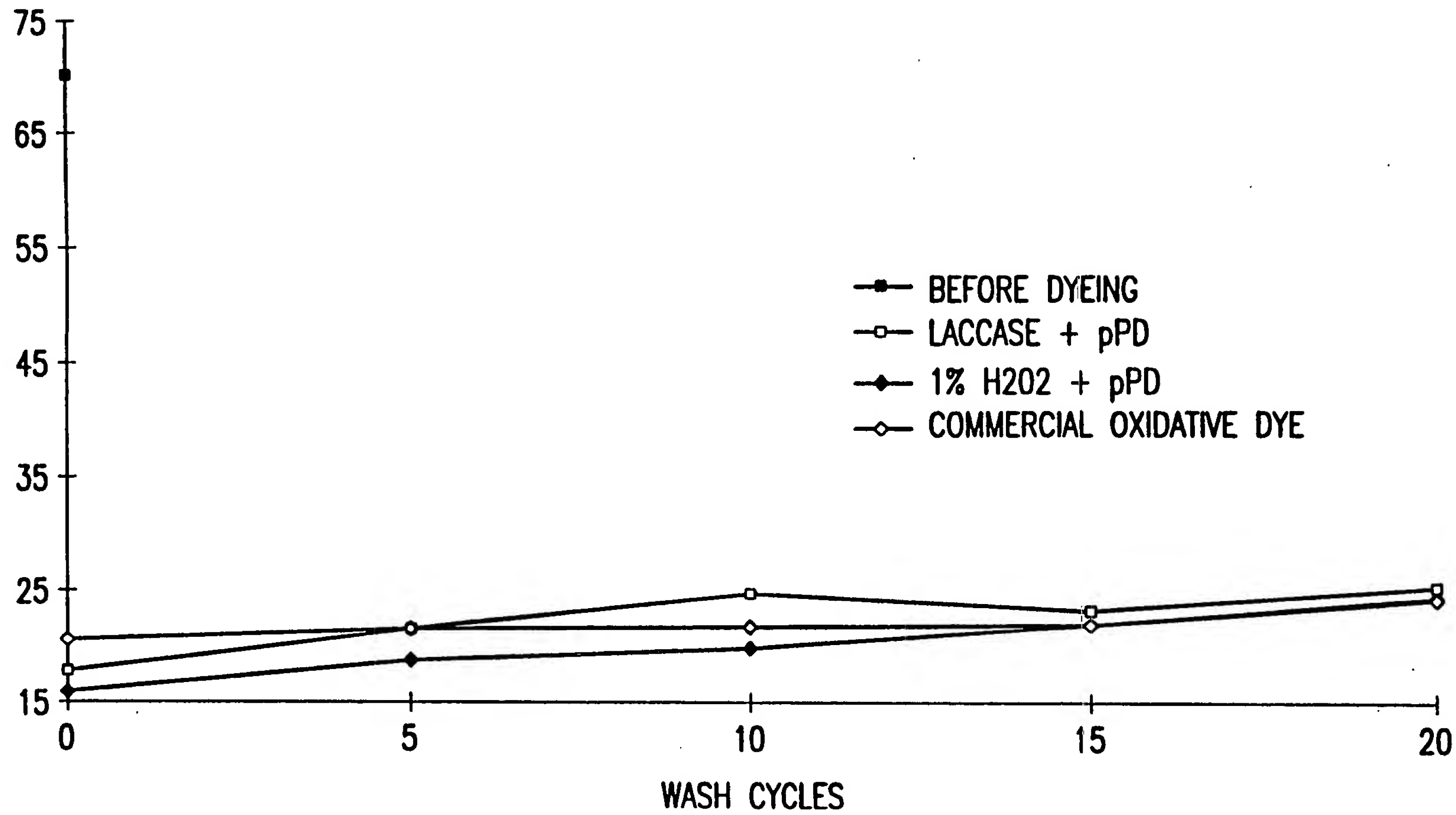

75
65
55
L* 45
35
25
15
BEFORE DYEING
LACCASE + pPD
1% H202 + pPD
COMMERCIAL OXIDATIVE DYE
0
5
10
15
20
WASH CYCLES

FIG.13

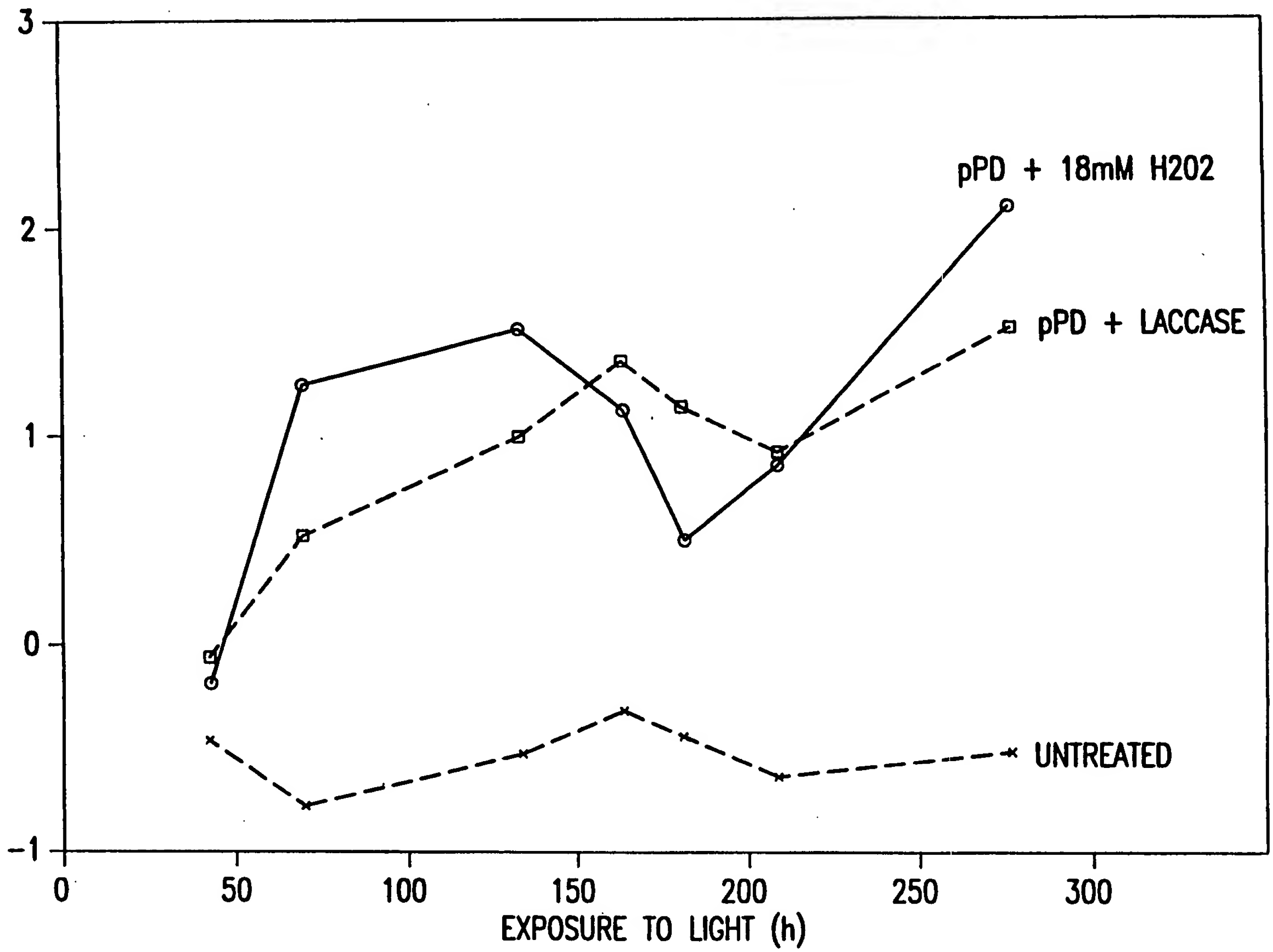

3
2
1
0
-1
0
50
100
150
200
250
300
EXPOSURE TO LIGHT (h)
pPD + 18mM H202
pPD + LACCASE
UNTREATED

FIG.14

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N9/02 C12N1/15 A61K7/13 A61K7/06
D21C5/00 C12N15/80 //(C12N1/15,C12R1:66)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K D21C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GEN. TECH. REP. NC (NORTH CENT. FOR EXP. STN.), vol. 175, 1994 pages 115-118, YAVER D.S. ET AL. 'The molecular cloning and expression of laccase genes from the white-rot basidiomycete Polyporus pinsitu' see the whole document	1-48
P,X	WO,A,95 01426 (NOVONORDISK AS ;SCHNEIDER PALLE (DK); PEDERSEN ANDERS HJELHOLT (DK) 12 January 1995 see page 6 - page 7; claim 22; example 2	15-17, 35-41, 45,48
X	DE,C,40 33 246 (PFLEIDERER UNTERNEMENSVERWALTUNG GMBH & CO.) 27 February 1992 see the whole document	15,16,35

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 October 1995	09.11.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Espen, J

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 48, no. 4, 1984 pages 849-854, BOLLAG J.-M. ET AL. 'Comparative studies of extracellular fungal laccases' see page 851; figure 2	15,35
A	DE,C,36 34 761 (HÜTTERMANN, A.) 18 February 1988 see the whole document	
A	LES COLLOQUES DE L'INRA, vol. 40, 1987 PARIS, pages 223-229, TROJANOWSKI A. ET AL. 'Solubilization and polymerization of lignin by several wood-inhabiting fungi' see the whole document	
A	MICROBIOS LETT., vol. 29, no. 113, 1985 pages 37-43, ILAN CHET ET AL. 'Decolourization of the dye Poly B-411 and its correlation with lignin degradation by fungi' see the whole document	

PCT/US 95/07536

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9501426	12-01-95	AU-B- 6924594	24-01-95
DE-C-4033246	27-02-92	NONE	
DE-C-3634761	18-02-88	EP-A- 0264076	20-04-88